



**PHD**

**Liver specific gene expression**

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# **Liver Specific Gene Expression**

Submitted by May Salem Al-Nbaheen

for the degree of PhD

of the University of Bath

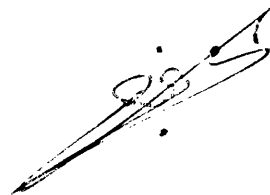
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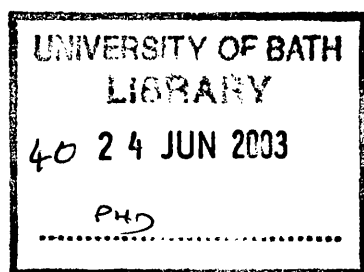
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***To Mam, and Dad,***

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## ABBREVIATIONS

AD I	Activation domain I
AD III	Activation domain III
AF-1	Activation function-1
AF-2	Activation function-2
AMPS	Ammonium persulfate
ApoC III	Apolipoprotein-C III
AP-1	Activate protein-1
AP-2	Activate protein-2
ATF4	Activating transcription factor 4
̢Fg	̢Fibrogen
̢gal	̢galactosidase
BSA	Bovine serum albumin
CCL <sub>4</sub>	Carbon tetrachloride
CdCl <sub>2</sub>	Cadmium chloride

Cd <sup>+2</sup> RE	Cadmium response element
C/EBP	CCAAT/enhancer binding protein
CMV	Cytomegalo virus
CO	Carbon monoxide
CPRG	Chlorophenol red β-galactopyranoside
DMEM	Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
dNTP	Deoxynucleotide triphosphate
DBP	D-binding protein
DNA	Deoxyribonucleic acid
EBNA-1	Epstein Barr Nuclear Antigen-1
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhancer green fluorescent protein
EPO	Eythropoietin
FACS	Fluorescence activated cell sorter
FBS	Foetal bovine serum
GCG	Genetics computer group
GFP	Green fluorescence protein
GH	Growth hormone
GST	Glutathione S-transferase genes
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HepG2	Human hepatocellular carcinoma
HIF-1	Hypoxia-inducible factor-1
HNF-1	Hepatocyte nuclear factor-1
HNF-3	Hepatocyte nuclear factor-3
HNF-4	Hepatocyte nuclear factor-4
HNF-6	Hepatocyte nuclear factor-6
HO	Heme oxygenase
HO-1	Heme oxygenase-1
HO-2	Heme oxygenase-2

HO-3	Heme oxygenase-3
HRE	Heme response element
HS	Hypersensitive site
HSE	Heat shock responsive element
HSV	Herpes simplex virus
HtTA	Human cervix carcinoma
ICR	Internal control region
IL-1	Interleukin-1
IL-6	Interleukin-6
LB	Luria Bertani
MEF	Myocyte enhancer factors
MEM-NEAA	MEM-non essential amino acids
MRE	Metal Regulatory Element
mRNA	messenger RNA
NaAs <sub>2</sub> O <sub>3</sub>	Sodium arsenite
NF-κB	Nuclear factor kappa B
NO	Nitric oxide
Nrf	Nuclear respiratory factor
<i>oriP</i>	origin of replication
OC	Onecut class
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Primer extension
PHF	Post-hepatectomy factor
PPAR-α	Peroxisome proliferator-activated receptor alpha
rpm	Revolution per minute
rRNA	ribosomal RNA
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S1	Endonuclease mapping
STAT-3	Signal transducer and activator of transcription 3

STAT-5	Signal transducer and activator of transcription 5
SV40	Simian virus 40
tRNA	transfer RNA
tTA	Trans activator
TBE	Tris-borate-EDTA buffer
TE	Tris.Cl-EDTA buffer
TEF-1	Transcription enhancer factor-1
TF	Transcription factor
TNF- $\alpha$	Tumor necrosis factor- $\alpha$
UBF	Upstream binding factor
UCE	Upstream control element
USF	Upstream stimulatory factor
UVA	Ultraviolet A

## ABSTRACT

One way of targeting gene expression *in vivo* is to control transcription using a tissue-specific regulatory system. Tissue-specific promoters or enhancers are in use in transgenic animals and could be utilized in medicine for gene therapy. At present the usual method for selection of a tissue-specific promoter is to identify a gene, which is expressed at unusually high level in the target tissue, and then to use the promoter for this gene to drive expression of another therapeutic gene in the target tissue. This approach is logical but does not always lead to high levels of gene expression. A second approach is to investigate the scope for discovery of synthetic specific promoters using a target tissue. In the present thesis we used both approaches to design plasmid DNA expression vectors that would carry liver-specific promoter/enhancer linked to a reporter gene (i.e. luciferase). We then transfected these vectors to both liver-derived and non-liver cell lines and then evaluated the liver-specificity of each construct by measuring the basal level expression of the reporter gene (i.e. luciferase activity) in both cell lines. The first part of this thesis dealt with literature survey using gene bank database, Vector NTI program software, and TFSEARCH algorithms to identify some of the transcription factors that are highly expressed in the liver. The research revealed the presence of four families of liver-enriched transcription factors, which are hepatocyte nuclear factor-1 (HNF-1), the CCAAT/enhancer binding protein (C/EBP), the hepatocyte nuclear factor-3 (HNF-3) and the hepatocyte nuclear factor-4 (HNF-4). Once this phase of the project has been completed, the HNF-4 was selected from the above group of liver-enriched transcription factors and used to design new synthetic

enhancers by inserting a tandem array of 1, 3 or 5 repeats of the HNF-4 binding site upstream of the SV40 promoter linked to the luciferase reporter gene within an Epstein-Barr virus (EBV)-based vector, p706. The results of transfection revealed that unexpectedly the HNF-4 binding sites in these constructs act as a repressor rather than enhancer of the liver-specific expression of the luciferase gene. The second approach was to use the promoter region of the liver-enriched heme oxygenase 1 (HO-1) gene to drive the expression of the luciferase reporter gene to mimic a therapeutic gene in the target tissue. The strategy involved the identification of the *cis*-acting elements that regulate basal human HO-1 gene expression by using EBV-based reporter plasmids containing deletions within the specific regions of 4.7 kb of the human HO-1 5'-flanking region linked to the luciferase gene. Basal level expression of these constructs was measured following transient transfection in both liver-specific HepG2 (human hepatoma) and non-liver HtTA-1 (Hela-derivative) cell lines. From these investigations, it was found that the expression of the luciferase reporter in 4.7 kb of the 5'-flanking region of the human HO-1 is preferentially higher in HepG2 cells when compared to the non-liver cell line HtTA-1, so that the 5'-flanking region of the HO-1 gene can be used for the design of liver-specific gene delivery systems. Furthermore one positive regulatory region at position -4080 to -3650 was identified that specifically functioned in HepG2 cells but not in HtTA-1 cells. A negative regulatory region was also found at position -380 to +1 that only functioned in HepG2 cells. There are many putative regulatory elements in these positive and negative regulatory regions that need further investigations. An analysis of the features of the 5'-flanking



region of the human HO-1 gene should provide pointers to a better understanding of the mechanism of tissue preferential expression and tissue-specific targeted gene delivery.

# CHAPTER 1 INTRODUCTION

## ***1.1. Gene Expression***

A gene is a repository of information, that holds the instructions for making one of the key molecules of life i.e. RNA. The sequence of bases in the RNA molecule is dependent on the sequence of bases in the gene (Weaver *et al.*, 1997). Thus, most genes are essentially blueprints for the building of individual cellular constituents. The coordinated program of protein synthesis from the genetic blueprint and the means of controlling its execution are called gene expression. Gene expression involves two stages for producing protein from information in a DNA gene (Lewin, 1997). The first step is the synthesis of RNA, which is complementary to one of the strands of DNA, this is called transcription. The second step is translation, where the information in the RNA is used to make a protein (Weaver *et al.*, 1997).

### **1.1.1. Regulation of Transcription in Prokaryotes**

The transcription unit is a segment of DNA (template) that is transcribed into RNA. Transcription occurs in three stages initiation; elongation and termination to synthesise RNA on a template of DNA. **Figure 1.1.** shows the process of transcription and the gene associated with various non-transcribed DNA elements such as promoters, enhancers and RNA polymerase (Adams *et al.*, 1986).

The RNA polymerase enzyme recognises a region called a “promoter”, which lies just upstream from the gene. The RNA polymerase binds tightly to the promoter region and causes separation of the two DNA strands within the promoter region. (initiation). Then the polymerase starts building the RNA chain (elongation).

During the elongation stage of transcription the enzyme moves along the DNA extending the growing RNA chain.

Finally, in the termination stage the terminators in conjunction with RNA polymerase loosens the association between the RNA product and the RNA polymerase and DNA transcription is terminated (Hawkins, 1996).

(1) Initiation:

(a) RNA polymerase binds to promoter:



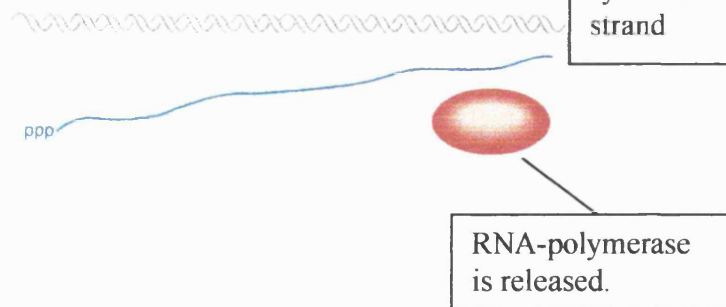
(b) First phosphodiester bond forms:



(2) Elongation:



(3) Termination:



**Figure 1.1.** Transcription processes with three stages initiation, elongation and termination to synthesis RNA from a template of DNA (adapted from Weaver *et al.*, 1997).

### **1.1.2. Regulation of Transcription in Eukaryotes**

Transcription in Eukaryotic cells is divided into three classes. A different RNA polymerase within the nuclei of cells transcribes each class:

- RNA polymerase I transcribes ribosomal RNA "rRNA".
- RNA polymerase II transcribes messenger RNA "mRNA".
- RNA polymerase III transcribes transfer RNA "tRNA" (Lewin, 1997).

### **1.1.3. Promoter**

A promoter element is a region of DNA involved in the binding of RNA polymerase to initiate transcription.

Since there are three different RNA polymerases in eukaryotic nuclei, so there are three different kinds of promoters. RNA polymerase I and II recognise one kind of promoter each, but RNA polymerase III uses two different classes of promoters.

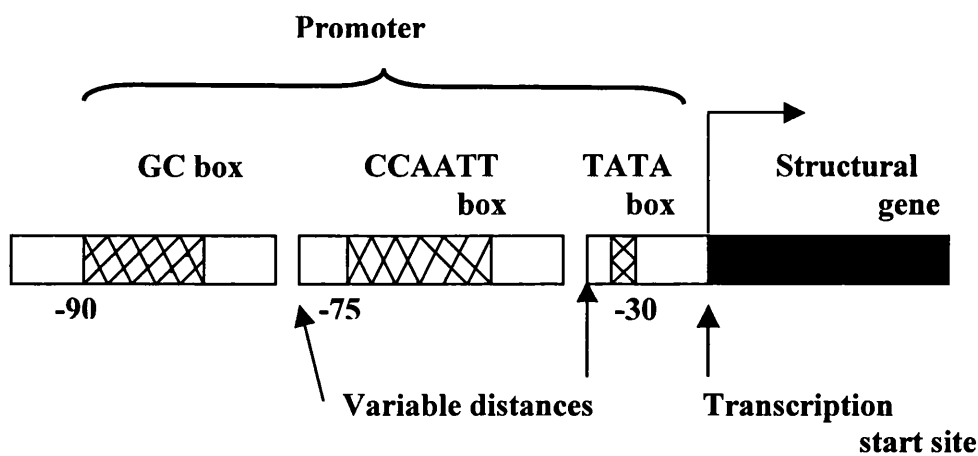
Those promoters rely on proteins called transcription factors, which are not themselves part of the RNA polymerase. There are two classes of transcription factors

(a)- General transcription factors and (b)- Gene-specific transcription factors called "gene activators" (Paule, 1981; Lewis *et al.*, 1982). These classes are discussed in detail in section 1.1.4.1. and 1.1.4.2.

#### **1.1.3.1. Promoters Recognised by RNA Polymerase II**

The promoters for RNA polymerase II consist of a number of sequence elements required for accurate and efficient initiation of transcription (Chambon *et al.*, 1984).

One important element is the **TATA box**: This is an A-T rich sequence element with the consensus sequence TATAAAA, found closest to the transcription start site, approximately at position -30 (see **Figure 1.2.**) and its function is to fix the location of the transcription start site (Adams *et al.*, 1986).



**Figure 1.2.** Diagram of the promoter region for RNA polymerase II (adapted from Adams *et al.*, 1986).

Another promoter element recognized by RNA polymerase II is the **CCAAT box**, found about fifty base pairs further upstream of the start site, approximately at position -75. This box has the consensus sequence of GGCCAATCT and it functions as part of the promoter in promoting efficient initiation.

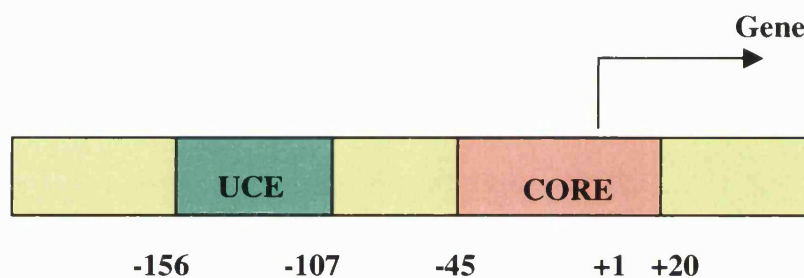
Another upstream promoter element, called the enhancer element is a 25 bp sequence that is repeated three times at position -90 bp just upstream from the TATA box. Each of these 25 bp repeats contains two copies of **GC box** with the sequence GGGCGG (Glick *et al.*, 1998) as shown in **Figure1.2.**

### 1.1.3.2. Promoter Recognised by RNA Polymerase I

RNA polymerase I transcribes only the genes for rRNA, from a single type of promoter. The rRNA promoter in human cells consists of two important DNA elements (Lewin, 1997; Hawkins, 1996).

(a)-The **core element** that is located between positions  $-45$  and  $+20$  and is sufficient for transcription to initiate.

(b)-The upstream control element (**UCE**) that is located between position  $-156$  and  $-107$  and is required for efficient transcription (**Figure 1.3**).



**Figure 1.3.** The human rRNA promoter, which consists of two DNA elements, UCE (green) and CORE (red), both of which are important for efficient transcription by RNA polymerase I.

### 1.1.3.3. Promoters Recognised by RNA Polymerase III

The promoters for the genes transcribed by RNA polymerase III do not lie in the 5'-flanking sequences of the gene but within the coding region of the gene itself. This region lies between positions  $+50$  and  $+83$  of the transcribed sequence. The promoter is known as an internal control region "**ICR**" (Korn, 1982; Hall *et al.*, 1982).

### **1.1.4. Transcription Factors**

Transcription of eukaryotic genes depends not only on RNA polymerase binding to a promoter but also on a collection of *trans*-acting proteins called transcription factors (TF) that interact with promoters and enhancers. They appear to be important for the formation of a stable initiation complex with the template and the accurate initiation of transcription (Adams *et al.*, 1986).

There are two classes of such factors: general transcription factors, and gene specific transcription factors (Weaver *et al.*, 1997).

#### **1.1.4.1. General Transcription Factors**

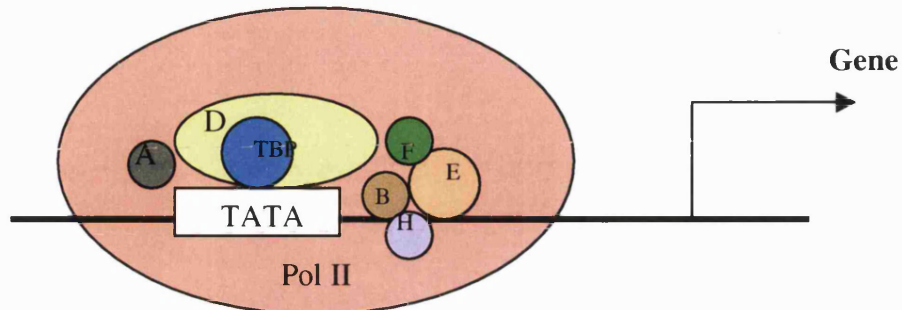
##### **1.1.4.1.1. RNA Polymerase II Transcription Factors**

The general transcription factors that are involved in transcription of most polymerase II genes interact with the region that extends upstream from the TATA box sequence (Hawkins, 1996).

The general transcription factors are: TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH.

In higher eukaryotes including mammals the TFIID contains several polypeptide subunits as TATA box-binding protein “TBP” or TATA box-binding protein associated factors “TAF’s”.

The preinitiation complex shown in **Figure 1.4.** is primed to initiate transcription and contains the promoter with all factors plus RNA polymerase II bound (Lewin, 1997).



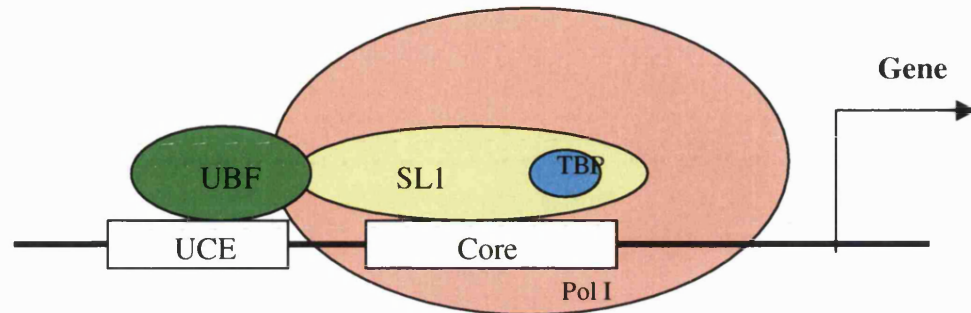
**Figure 1.4.** The class II preinitiation complex of RNA polymerase II, containing TFIIA (A), TFIIB (B), TFIID (D), TFIIIE (E), TFIIF (F), and TFIIH (H) bound to the TATA-box and TBP (adapted from Weaver *et al.*, 1997).

#### 1.1.4.1.2. RNA Polymerase I Transcription Factors

RNA Polymerase I requires two transcription factors:

- (a)- Upstream binding factor “**UBF**” that binds in a sequence-specific manner to related sequences in the core promoter.
- (b)-“**SL1**” that does not by itself have specificity for the promoter, but once UBF1 has bound, SL1 can bind to the region of DNA. Once both factors are bound to form the preinitiation complex, RNA polymerase I can bind to the core promoter to initiate transcription as shown in **Figure 1.5.** (Weaver *et al.*, 1997).





**Figure 1.5.** The class I preinitiation complex of RNA polymerase I. The two parts of the rRNA promoter (UCE and Core) are represented by white boxes (adapted from Weaver *et al.*, 1997).

#### 1.1.4.1.3. RNA Polymerase III Transcription Factors

Three transcription factors are involved in transcription by RNA polymerase III:

TFIIIA, TFIIIB and TFIIIC. TFIIIA is a member of an interesting class of zinc finger proteins, TFIIIB is the true transcription initiation factor for RNA polymerase III and consists of TBP and two other proteins. TFIIIC is a large protein complex (>500 KD) bound to the internal promoter and by protein-protein interaction, allows TFIIIB to bind near to the transcription start site (Glick *et al.*, 1998).

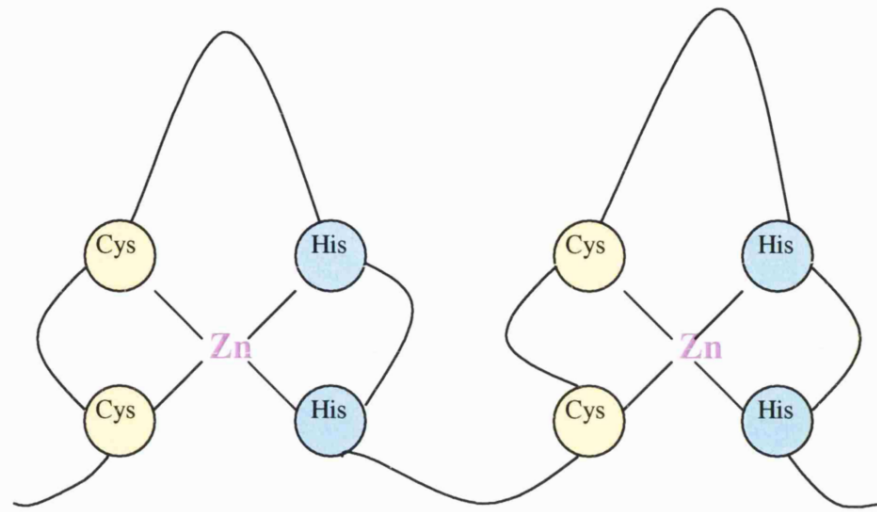
#### 1.1.4.2. Gene-Specific Transcription Factors

In addition to the general transcription factors there are a group of gene-specific factors. Three main types of protein structures are involved in interactions of the

transcription factor with the DNA (Hawkins, 1996). These are “Zinc fingers”, “Helix turn helix”, and “Leucine zippers”.

#### **1.1.4.2.1. Zinc Fingers**

These are sequence of about 9-13 amino acid residues predicted to be finger-shaped because each of these domains binds zinc ion as shown in **Figure 1.6**. The zinc finger binds to the major groove of DNA and interacts with about 5 bases of the template. The specific binding of these proteins to particular sites on DNA is determined by certain residues either in the fingers or immediately adjacent to them (Hawkins, 1996; Weaver *et al.*, 1997).



**Figure 1.6.** Schematic diagram of two adjacent zinc fingers, each finger is bound to four amino acids: two cysteines (yellow) and two histidines (blue), holding the finger in the correct conformation for DNA binding (adapted from Weaver *et al.*, 1997).

#### 1.1.4.2.2. Helix-Turn-Helix

This is another structural motif commonly found in DNA binding proteins in both prokaryotes and eukaryotes. It consists of  $\alpha$ -helix containing eight amino acid residues. The proteins containing this motif are frequently on one side of the helix making Van der Waals contacts with the bases.

#### **1.1.4.2.3. Leucine Zipper: 'bZIP domains'**

In the leucine zipper the DNA binding proteins are  $\alpha$ -helices containing a leucine residue in every seventh position. There are generally four leucine residues on each helix and this structure has been christened a "Leucine Zipper" (Alberts *et al.*, 1989). This motif is described in more detail in section 1.1.5.1.5.

#### **1.1.5. Tissue/Organ Enriched Transcription Factors**

Most of the proteins accounting for organ functions are expressed mainly or exclusively within a specific set of tissue-specific cells which therefore provide an attractive model for studying tissue specific gene expression (Tronche *et al.*, 1992).

Present knowledge of transcriptional regulation in organs has been derived from the study of two regulatory elements, the analysis of promoter and far-upstream enhancers of genes selectively expressed in adult tissues using transient transfection in tissue cell lines. The binding sites of the organ-enriched and ubiquitous transcription factors for these two elements are clustered within short regions however, transcription of a given gene in adult tissues appears to require a particular combination of multiple transcription factor binding sites which are defined as combinatorial controls (Cereghini, 1996). Some of these nuclear factors controlling gene expressed in adult tissue, are involved in the early events of organ differentiation and development. Such factors therefore play critical roles in maintaining the expression of organ-specific functions in the adults.

Organ/tissue specific gene expression has been recently used as a tool for development of organ/tissue specific gene therapy protocols. Such gene therapy employs well-understood specific transcription factor binding elements as building blocks for generation of organ/tissue-specific synthetic promoters. For example

skeletal muscle is an attractive target for somatic gene therapy because of its long life span, ease of accessibility for intramuscular injections and large capacity for protein synthesis and secretion (Tripathy *et al.*, 1996; Hasse, 1997). However, relatively low levels of expression from naturally occurring promoters have limited the use of muscle as a gene therapy target. To counteract this limitation, recently Draghia-Akli and coworkers (1999) have developed a strategy to isolate synthetic muscle-specific promoters that will have both high *in vitro* and *in vivo* transcriptional activity. The strategy involved random assembling of several myogenic regulatory elements i.e. serum response element “SRE”, myocyte-specific enhancer binding factors “MEF-2”, “MEF-1” and transcription enhancer factor-1 “TEF-1” into synthetic promoter recombinant libraries. This was followed by the screening of the libraries that resulted in isolation of several artificial novel promoters whose transcriptional potencies in terminally differentiated muscle greatly exceeded those of natural myogenic and viral gene promoters (Li *et al.*, 1999).

Another potential target for organ-specific gene therapy is the liver. This has led to extensive study of the hepatic transcription regulatory network notably the liver-enriched transcription factors. Indeed experiments using cDNA library from mouse liver poly(A)+ RNA that was then differentially screened with poly(A)+ RNA from liver and non-liver cells provided strong evidence that the predominant control of liver-specific gene expression resides at the level of transcription (Derman *et al.*, 1981; Aran *et al.*, 1995). Clones proven to be liver-specific were picked and used as templates for hybridization with radioactive RNA newly transcribed *in vitro* in nuclei isolated from liver and non-liver tissues. The hybridization signals obtained with RNA synthesized with liver nuclei were at least 10 times more intense than

those obtained with nuclei from other tissues. Because the cDNA clones represented an unbiased population of transcripts, the findings led to the conclusion that liver-specific gene expression is primarily a consequence of transcriptional regulation (Derman *et al.*, 1981).

Since the key proteins for liver function are expressed mainly in hepatocytes, much attention has been given to understand the mechanism of transcription regulatory network in these hepatic cells. The transcription rate of genes encoding liver-specific proteins is distinctly higher in hepatocytes as compared with other cell types (Powell *et al.*, 1984). Furthermore, the transcription of several hepatic genes is specifically activated in hepatocytes during liver development and later modulated depending on extracellular stimulation (Schmid and Schulz, 1990; Shiojori *et al.*, 1991).

#### 1.1.5.1. Liver Enriched Transcription Factors

Transient transfection assays in which the introduced gene does not integrate into the genome have been instrumental in identifying the regulatory sequences in DNA that confer liver-specific gene expression. Analyses performed on a wide variety of genes that code for entirely different proteins show shared regulatory sequences. Moreover, characterization of the regulatory sequences of a number of genes has shown that each gene contains a combination of some or all of the liver-specific shared motifs (Benvenisty and Reshef, 1991; Aran *et al.*, 1995). It is this combination of *cis*-regulatory elements rather than a single element that appears to be required for liver-specific gene expression. Finally, these shared motifs bind

distinct cognate liver-enriched transcription factors and have aided in isolating and characterizing these factors (De Simone and Cortese, 1991).

Over the last few years a number of transcription factors have been identified that have important roles in regulating liver development and differentiation. These specific transcription factors use novel mechanisms for gene expression that ultimately direct cell differentiation (Duncan, 2000).

So far, six families of liver-enriched transcription factors were identified that participate in the expression of liver genes in the adult hepatocytes i.e. hepatocyte nuclear factor-1 “HNF-1”, hepatocyte nuclear factor-3 “HNF-3”, hepatocyte nuclear factor-4 “HNF-4”, hepatocyte nuclear factor-6 “HNF-6”, CCAAT/enhancer binding protein “C/EBP”, and D-binding protein “DBP” (Cereghini, 1996; Schrem *et al.*, 2002). The analysis of the tissue distribution of these factors and the determination of their hierarchical relations have led to the hypothesis that the cooperation of liver-enriched transcription factors with the ubiquitous transactivating factors is necessary, and possibly even sufficient, for the maintenance of liver-specific gene transcription (Hayashi *et al.*, 1999).

#### **1.1.5.1.1. Hepatocyte Nuclear Factor-1 “HNF-1”**

Hepatocyte nuclear factor-1 (HNF-1) mRNA and proteins although enriched in liver, can also be found in kidney tissues, intestine, and pancreas at significant different levels (Rey-camos *et al.*, 1991). HNF-1 is composed of HNF-1 $\alpha$  and HNF-1 $\beta$  hetero- and homo-dimers. These homeoproteins share identical DNA-binding domains but have different transcriptional activation properties (Kuo *et al.*, 1991; Song *et al.*, 1998). Interestingly, HNF-1 $\alpha$ , but not HNF-1 $\beta$ , is expressed in the liver. Under physiologic conditions as well as in transfection experiments with

HNF-1 $\alpha$  and HNF-1 $\beta$ , stable homodimer formation can be found in the liver, whereas in other organs, heterodimers are also detected. From these data it is assumed that the extent of heterodimerization might be regulated in a tissue-specific manner. Furthermore, it could be shown that exclusive expression of HNF-1 $\beta$  is associated with repression of a subset of hepatocyte-specific genes in the dedifferentiated hepatocyte cell line C2, in differentiated F9 cells, in somatic hybrids between hepatocytes and fibroblasts, and in the lung (Mendel *et al.*, 1991a).

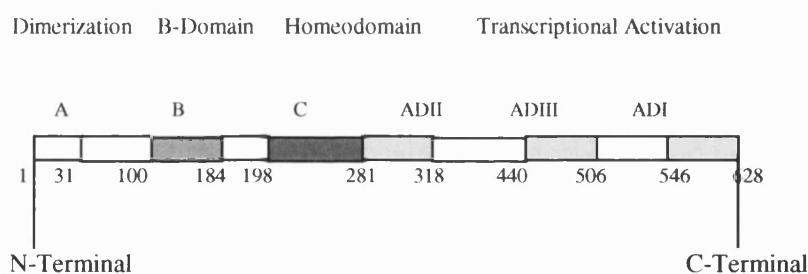
HNF-1 $\alpha$  is one of the first liver-enriched transcription factor, to be identified by its interaction with an essential sequence for liver-specific transcription of the  $\beta$ -fibrinogen, albumin, and  $\alpha$ 1-antitrypsin promoters (Hardon *et al.*, 1988). The binding site of HNF-1 $\alpha$  was found in more than 30 different liver-specific genes with the consensus sequence of 15 bp containing an inverted repeat of GGTAAATNATTAAC.

The genes regulated by HNF-1 $\alpha$  encode products involved in diverse metabolic functions, including synthesis of seroproteins, carbohydrate metabolism, and detoxification (Cereghini, 1996). For example HNF-1 $\alpha$  is one of the most important transactivators of liver-specific albumin transcription (Maire *et al.*, 1989). It also acts as an accessory factor to enhance the inhibitory action of insulin on mouse glucose-6-phosphatase gene transcription (Streeper *et al.*, 1998). HNF-1 $\alpha$  is also an accessory factor required for activation of glucose-6-phosphatase gene transcription by glucocorticoids (Lin *et al.*, 1998). Several lines of evidence point to a direct transactivation of the mouse ferrochelatase promoter by HNF-1 $\alpha$  in the liver (Muppala *et al.*, 2000).

The structure of HNF-1 $\alpha$  can be described as a homeodomain-containing transcription factor that is located within its first 281 amino acids. It is unique



among the vertebrate homeodomain-containing proteins in that it dimerizes in the absence of its DNA recognition sequence (Mendel *et al.*, 1991b). It is characterized by a tripartite structure, in which the amino acids 1 to 31 are necessary and sufficient for dimerization of protein, as shown in **Figure 1.7**. This tripartite structure is composed of an amino-terminal dimerization domain (domain A), specific domain conferring a specific sequence (domain B) and a typical homeodomain structure (domain C). HNF-1 $\alpha$  requires transactivation to the carboxy-terminal region and in *ex vivo* situation, this appears to be achieved by the combined action of two regions: The activation domain I (ADI) rich in serine and the activation domain III (ADIII) rich in glutamine (Ceska *et al.*, 1993).



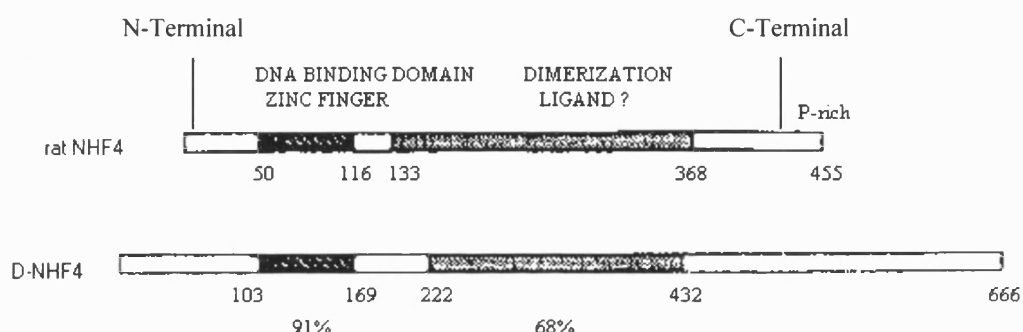
**Figure 1.7.** Schematic diagram representation of the structure of HNF-1 $\alpha$  transcription factor.

#### 1.1.5.1.2. Hepatocyte Nuclear Factor-4 “HNF-4”

Hepatocyte nuclear factor-4 (HNF-4) is a liver-enriched transcription factor but it is also expressed at significant levels in primary blastocyst endoderm, intestinal and kidney cells (Hall *et al.*, 1995). It belongs to the zinc finger

family. It is a member of the steroid hormone receptor superfamily, and it binds DNA as a dimer (Plengvidhya *et al.*, 1999).

The carboxyl terminal region of HNF-4 contains a large hydrophobic region “133-373” reminiscent of the dimerization and ligand binding domain of other receptors (Cereghini, 1996) as shown in **Figure 1.8**.



**Figure 1.8.** Comparison of conserved region of HNF-4, between rat HNF-4 and the *Drosophila* homologue HNF-4 (D).

In addition, HNF-4 contains a zinc finger DNA binding domain at position 50 to 116. HNF-4 possesses a proline-rich region at the carboxyl terminus and three serine-threonine regions located throughout the molecule. HNF-4 is phosphorylated at tyrosine, serine and to a lesser extent at threonine residues (Cereghini, 1996). The binding site of this factor i.e. KGCWARGKYCAY is considered as an activator of the gene apolipoprotein-C III (ApoC III) in humans. However, in the rat the gene activator is HNF-1. The composition of the binding site of this human factor between position “-82 to -69” is as

follows: TGACCTTTGCCAG (Kel *et al.*, 1995; Tian *et al.*, 1991). Such binding site is required for the hepatoma-specific expression of HNF-1 $\alpha$ , suggesting its participation in liver development.

HNF-4 is also involved in the regulation of several genes such as those encoding for pyruvate kinase, the blood clotting factor (factor IX), medium chain acyl-coenzyme A dehydrogenase, tyrosineamine-transferase, ornithine transcarboxylase, cellular retinal-binding protein II, transferrin, ApoA I, ApoA II, ApoB I, and ApoC III (Hall *et al.*, 1995; Tian *et al.*, 1991; and Mietus-snyder *et al.*, 1992). It is also involved in the expression of the hepatitis B virus enhancer, the  $\alpha$ 1-antitrypsin and transthyretin genes (Flodby *et al.*, 1993; Costa *et al.*, 1989).

HNF-4 contains two transactivation domains, designated activation function-1 (AF-1) and activation function-2 (AF-2), which activate transcription in a cell type-independent manner. Deletion of AF-1 results in 40% reduction of the HNF-4-mediated activation (Hadzopoulo-Cladaras *et al.*, 1997). HNF-4 binds to the AF-1 portion of the region between “-81 to -62”. AF-1 is considered as a liver enriched transcription factor, that binds to a region of the ApoB promoter “-81 to -52”, which is critical to the expression of phosphoenolpyruvate carboxy kinase gene in hepatocytes.

Studies with *in vitro* translated HNF-4 protein show that it binds to its recognition site as a dimer, and cotransfection assays indicate that it activates transcription in a sequence-specific fashion in non-hepatic (Hela) cells (Sladek *et al.*, 1990).

HNF-4 mRNA and protein level are not affected in rat liver after partial hepatectomy (Flodby *et al.*, 1993). However, following hepatectomy some

cytokines such as tumor necrosis factor (TNF- $\alpha$ ), interleukin-1 (IL-1) and interleukin-6 (IL-6) are believed to induce HNF-4 factor, and other factors such as PHF (post-hepatectomy factor)/(nuclear factor kappa B) NF- $\kappa$ B and STAT-3 (signal transducer and activator of transcription 3) (Diehl *et al.*, 1996).

HNF-4 subfamily members include HNF-4 $\alpha$ , HNF-4 $\beta$  and HNF-4 $\gamma$ :

- **HNF-4 $\alpha$**  (gene symbol, TCF14) is believed to play an important role in maintaining hepatocyte specific differentiation. It is a positive regulator and activator of HNF-1 $\alpha$  expression (Yamagata *et al.*, 1996), and it is expressed at the earliest stage of liver formation.

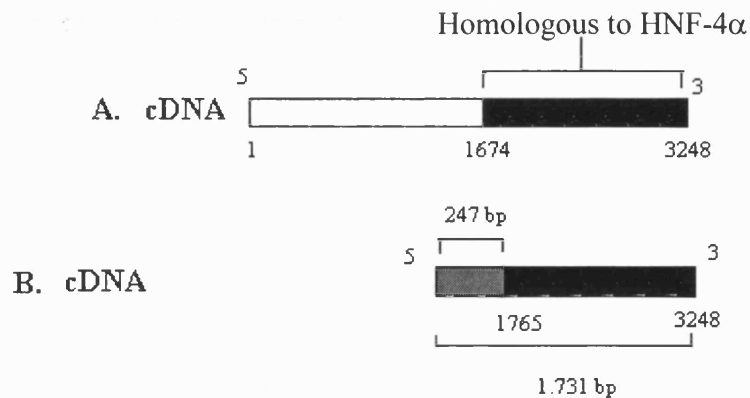
Moreover, HNF-4 $\alpha$  is expressed in the mammalian kidney and digestive tract (Sladek *et al.*, 1990; Holew *et al.*, 1997). Disruption of the HNF-4 $\alpha$  gene leads to an early embryonic lethal phenotype associated with a failure of differentiation of the visceral endoderm (Spath *et al.*, 1997).

HNF-4 $\alpha$  is expressed in high levels during the liver development and differentiation phase in mice (Duncan *et al.*, 1994).

Further complexity of gene control by HNF-4 $\alpha$  transcription factors can be anticipated by the differential splicing of the 10 initially identified exons of the HNF-4 $\alpha$  gene (Nakhei *et al.*, 1998). Thus, so far, seven distinct splice variants have been identified in human and murine cDNA samples. HNF-4 $\alpha$ -1 represents the initially identified transcript, whereas HNF-4 $\alpha$ 2 through HNF-4 $\alpha$ 7 are the splice variants identified subsequently (Kritis *et al.*, 1996; Furuta *et al.*, 1997; Nakhei *et al.*, 1998).

In 1998, Hertz and coworkers published the discovery of several ligands for HNF-4 with agonistic and antagonistic effects on HNF-4 $\alpha$  transcriptional activity. It could be demonstrated that long-chain fatty acids directly modulate the transcriptional activity of HNF-4 $\alpha$  by binding as their acyl-Coenzyme A thioesters to the ligand-binding domain of HNF-4 $\alpha$  (Hertz *et al.*, 1998).

- **HNF-4 $\beta$**  was first identified in *Xenopus* and showed distinct activation and expression profiles in oogenesis and embryogenesis of *Xenopus laevis* (Holew *et al.*, 1997).
- **HNF-4 $\gamma$**  is highly homologous to HNF-4 $\alpha$  showing almost complete amino acid identity in the DNA- and ligand-binding domains as shown in **Figure 1.9**. (Drewes *et al.*, 1996). However Northern blot analyses reveal that HNF-4 $\gamma$  is expressed in the kidney, pancreas, small intestine, testis and colon but not in the liver, whereas HNF-4 $\alpha$  mRNA is found in all of these tissues (Drewes *et al.*, 1996).



**Figure 1.9.** Schematic representation of the HNF-4 $\gamma$  cDNAs. The black boxes represent the cDNA regions homologous to the HNF-4 $\alpha$  cDNA (A and B). The gray box represents the new 5' sequence (B).

Recent studies have demonstrated that HNF-4 could also act as a negative regulator of several mammalian genes such as mitochondrial HMG-Coenzyme A synthase, acetyl-Coenzyme A oxidase, peroxisome proliferator-activated receptor alpha (PPAR- $\alpha$ ) and rat arginase (Rodriguez *et al.*, 1998; Nishiyama *et al.*, 1998; Chowdhury *et al.*, 1996).

#### 1.1.5.1.3. Hepatocyte Nuclear Factor-3 “HNF-3”

The Hepatocyte nuclear factor-3 (HNF-3)/forkhead (fkh) family contains a large number of transcription factors and folds into a winged helix motif (Marsden *et al.*, 1998; Jin *et al.*, 1999). The HNF-3 gene subfamily is composed of three proteins ( $\alpha$ ,  $\beta$  and  $\gamma$ ) that mediate hepatocyte-enriched transcription of numerous genes

whose expression is necessary for organ function (Samadani and Costa, 1996). All three transcription factors share strong homology in the winged-helix/fork head DNA-binding domain (region I) that overlaps with the nuclear localization signal (Qian and Costa, 1995). HNF-3 $\alpha$ , - $\beta$ , and - $\gamma$  are able to recognize the same DNA sequence (Samadani & Costa, 1996; Pani *et al.*, 1992 a,b). They also possess two similar stretches of amino acids at the carboxy terminus (regions II and III) and a fourth segment of homology at the amino terminus (region IV) (Pani *et al.*, 1992a,b).

The HNF-3 proteins demonstrate homology with the *Drosophila* homeotic gene 'fkh' in regions I, II and III, suggesting that HNF-3 may be its mammalian homologue (Pani *et al.*, 1992a). Experiments using site-directed mutagenesis within regions II and III (amino acids 361-458) of HNF-3 $\beta$  demonstrated their importance for transactivation. In cotransfection assays with expression vectors that produced different truncated HNF-3 $\beta$  proteins, amino-terminal sequences defined by conserved region IV also contributed to transactivation, but region IV activity required the participation of the region II-III domain (Pani *et al.*, 1992a).

A number of liver-specific genes that are predominantly expressed in the liver are positively regulated by HNF-3 $\alpha$ , - $\beta$ , or - $\gamma$  through interaction with the respective *cis*-acting HNF-3-binding elements in the promoters of these genes. Examples of such genes include albumin (Liu *et al.*, 1991), aldolase B (Gregori *et al.*, 1993),  $\alpha$ 1-antitrypsin (Samadani and Costa, 1996),  $\alpha$ 2-macroglobulin (Samadani and Costa, 1996), apolipoprotein B (Brooks *et al.*, 1991), insulin-like growth-factor binding protein 1 (O'Brien *et al.*, 1995), transthyretin prealbumin (Qian and Costa, 1995) and transferrin (Stenson *et al.*, 2000). In contrast, HNF-3 bound to the HNF-3 binding site of the human aldolase B promoter completely antagonizes

transactivation of the liver-specific aldolase B gene by HNF-1 and DBP (Gregori *et al.*, 1993).

Partial hepatectomy produces minimal fluctuation in HNF-3 ( $\alpha$ ,  $\beta$  and  $\gamma$ ) and transthyretin expression, suggesting that HNF-3 $\alpha$ , - $\beta$  and - $\gamma$  expression is not influenced by proliferative signals induced during liver regeneration. In acute-phase livers a dramatic reduction in HNF-3 $\alpha$  expression is observed, which correlates with a decrease in the expression of target genes, such as the transthyretin gene (Qian *et al.*, 1995).

HNF-3 $\gamma$  is an important regulator of liver-specific genes, and the expression of this factor is reduced in the liver injured by the toxin carbon tetrachloride (CCl<sub>4</sub>) (Nakamura *et al.*, 1999). HNF-3 $\gamma$  is thought to be involved in anterior-posterior regionalization of the primitive gut. In the HNF-3 $\gamma$  locus, 170 kb contain all the elements important in the regulation of HNF-3 $\gamma$ . A 3'-enhancer could be identified that contains a HNF-1 $\alpha$  and - $\beta$ -binding site that was shown to be crucial for enhancer function *in vitro* (Hiemisch *et al.*, 1997).

Studies using embryoid bodies in which one or both HNF-3 $\alpha$  or HNF-3 $\beta$  genes were inactivated showed that HNF-3 $\beta$  was necessary for expression of HNF-3 $\alpha$ . HNF-3 $\beta$  positively regulated the expression of HNF-4 $\alpha$ /HNF-1 $\alpha$  and their downstream targets, implicating a role in diabetes. In these studies HNF-3 $\alpha$  acted as a negative regulator of HNF-4 $\alpha$ /HNF-1 $\alpha$ , demonstrating that HNF-3 $\alpha$  and HNF-3 $\beta$  have antagonistic transcriptional regulatory functions *in vivo*. HNF-3 $\alpha$  did not appear to act as a classic biochemical repressor but, rather, exerted its negative effect by competing for HNF-3-binding sites with more efficient activator HNF-3 $\beta$ . In addition, the HNF-3 $\alpha$ /HNF-3 $\beta$  ratio was modulated by the presence of insulin,



providing evidence that the HNF network may have important roles in mediating the action of insulin (Duncan *et al.*, 1998).

#### **1.1.5.1.4. Hepatocyte Nuclear Factor-6 “HNF-6”**

The Hepatocyte nuclear factor-6 (HNF-6) is a liver-enriched transcription factor that contains a single-cut domain and a novel type of homeodomain. Comparative trees of mammalian, *Drosophila*, and *C. elegans* proteins showed that HNF-6 defines a new class of homeodomain proteins called ‘onecut class’, OC (Lannoy *et al.*, 1998). Human OC-2, the second mammalian member of this class, is located on human chromosome 18. The distribution of OC-2 mRNA in humans is tissue-restricted, the strongest expression being detected in the liver and skin. The amino acid sequence of OC-2 contains several regions of high similarity to HNF-6. The recognition properties of OC-2 for binding sites present in regulatory regions of liver-expressed genes differ from, but overlap with, those of HNF-6. It might be that in the future, HNF-6 and OC-2 will be regarded as two members of a bigger family (Jacquemin *et al.*, 1999).

In 1998, Rastegar and coworkers have identified two rat cDNA species coding for two isoforms, HNF-6 $\alpha$  (465 residues) and HNF-6 $\beta$  (491 residues), which differed only by the length of the spacer between the two DNA binding domains (Rastegar *et al.*, 1998). Observation that HNF-6 contributes to the control of the expression of transcription factors and is expressed at early stages of liver, pancreas, and neuronal differentiation suggest that HNF-6 participates in several developmental programs (Landry *et al.*, 1997).

HNF-6 recognizes the -138 to -126 regions of the HNF-3 $\beta$  promoter. Site-directed mutagenesis of this HNF-6 site diminishes reporter gene expression, suggesting that HNF-6 activates transcription of this promoter and may thus play a role in epithelial cell differentiation of gut endoderm via regulation of HNF-3 $\beta$  (Samadani and Costa, 1996). Later, it was recognized that HNF-6 is required for HNF-3 $\beta$  promoter activity and that HNF-6 also recognizes the regulatory region of numerous liver-specific genes (Rausa *et al.*, 1997).

HNF-6 expression can be regulated by growth hormone (GH) (Lahuna *et al.*, 2000). In hypophysectomized rats, HNF-6 mRNAs increase within 1 h after a single injection of GH. The same GH-dependent induction could be reproduced on isolated rats (Rastegar *et al.*, 2000). It appears that GH stimulates transcription of the HNF-6 gene by a mechanism involving STAT-5 (signal transducer and activator of transcription 5) and HNF-4. Moreover HNF-6 appears to participate not only as an effector, but also as a target, to the regulatory network of liver transcription factors, and that several members of this network are GH-regulated (Lahuna *et al.*, 2000).

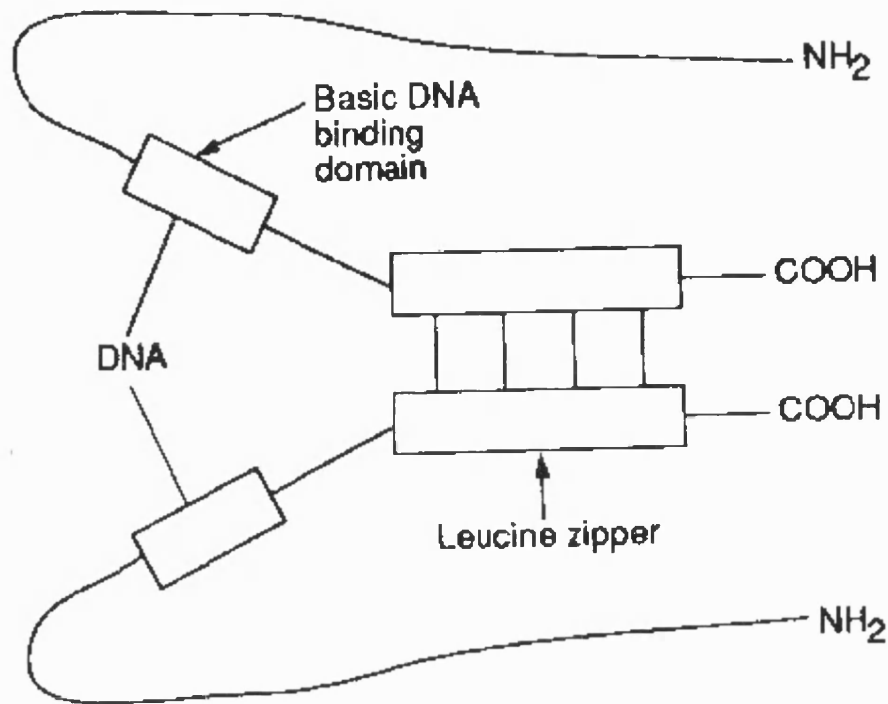
Recent studies from Lemaigre and coworkers (1999) reveal that HNF-6 inhibits the glucocorticoid-induced stimulation of two genes coding for enzymes of liver glucose metabolism, 6-phosphofructo-2-kinase and phosphoenol-pyruvate carboxykinase. The authors demonstrated that binding of HNF-6 to DNA is required for inhibition of glucocorticoid receptor activity. Furthermore both *in vitro* and *in vivo* experiments suggest that this inhibition is mediated by a direct HNF-6/glucocorticoid receptor interaction involving the amino-terminal domain of HNF-6 and the DNA-binding domain of the receptor (Pierreux *et al.*, 1999).

#### 1.1.5.1.5. CCAAT/Enhancer Binding Protein

CCAAT/enhancer binding protein (C/EBP) was first discovered in rat liver nuclei (Graves *et al.*, 1986). C/EBP proteins are four transcription factors described as C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , and C/EBP $\gamma$ . They are highly enriched in the liver but they are also found in fat cells (Kel *et al.*, 1995). The composite regulatory elements affecting gene transcriptions of C/EBP $\beta$  are as follows: CTGGGAAGATGTTGCTTA. It activates  $\beta$ -fibrogen ( $\beta$ -Fg) and IL-8 in human genes, but in the rat it activates the cytochrome-P450 (CYP2D5) gene, and in the mouse it activates the serum albumin gene (Kel *et al.*, 2000).

C/EBP belongs to the bZIP family. The bZIP motif is composed of 50-60 amino-acid residues. It consists of two distinct segments: the leucine zipper and the basic region (Landschulz *et al.*, 1989; Kim *et al.*, 1993). The leucine zipper mediates dimerization and symmetrically positions a divergent pair of basic-region  $\alpha$ -helices, through the major groove of each DNA half-site (Agre *et al.*, 1989).

The basic region (bZIP segment) undergoes a folding transition. The previously unfolding basic region becomes  $\alpha$ -helical and a quintet of conserved basic region residues are positioned to make contact with the DNA (Ellenberger *et al.*, 1992). Generally mutations causing replacement of the leucine residues in the leucine zipper prevent the combination of polypeptide chains to each other, whereas mutations in the basic region abolishes binding of the leucine zipper to DNA (Hawkins, 1996). It should be noted that the leucine zipper does not bind directly to DNA but it facilitates the dimerization of the protein. Such dimerization provides the correct protein structure for DNA binding, which is enriched in basic amino-acids therefore can interact directly with acidic DNA as shown in **Figure 1.10**.



**Figure 1.10.** Model for the structure of the Leucine Zipper and the adjacent DNA-binding domain following dimerization of the transcription factor C/EBP (adapted from Latchman, 1990).

(Latchman, 1990). bZIP proteins mediate a range of biological processes including segmentation, energy regulation, memory and oncogenesis (Cordes *et al.*, 1994).

Both C/EBP $\beta$ , and C/EBP $\delta$  are implicated in signal transduction as nuclear targets for cytokine induced gene expression in various dividing and non-dividing cell types.

C/EBP $\alpha$  has been shown to activate glutathione S-transferase genes (GST) in the rat. Furthermore, in the non-growing and active metabolizing normal liver, C/EBP $\alpha$

expression is high and restricted to hepatocyte-cells. However, in rapidly growing hepatoma cells its expression is low (Friedman *et al.*, 1989).

C/EBP $\alpha$  binds to the promoter of the albumin,  $\alpha$ 1-antitrypsin, alcohol dehydrogenase, and factor IX genes (Flodby *et al.*, 1993). In partial hepatectomy in rats C/EBP $\alpha$  has been shown to have a rapid down regulation at the transcriptional level, whereas the C/EBP $\beta$ , and C/EBP $\delta$  are induced (Flodby *et al.*, 1993). Furthermore, C/EBP is shown to control ApoB expression (Birkenmeire *et al.*, 1989).

Rastegar *et al.*, (2000) have shown that in hypophysectomized rats GH treatment leads to a rapid decrease in liver C/EBP $\alpha$  protein concentration at the time when HNF-6 mRNA is increased by the GH. This is followed by an increase in the C/EBP $\alpha$  mRNA and protein at the time when there is a decrease in the level of HNF-6 mRNA. The authors have concluded that both C/EBP $\alpha$  mRNA and protein levels are controlled by GH.

Studies by Mischoulon *et al.*, (1992) have shown that in partial hepatectomized rats C/EBP $\alpha$  mRNA levels decrease by 60-80% within 1-3 hours. It also decreases during the growth of freshly isolated normal hepatocytes in culture. The decrease in C/EBP $\alpha$  is shown to be blocked by cycloheximide. The drop in C/EBP $\alpha$  gene expression suggests that C/EBP $\alpha$  has an anti-proliferative role in highly differentiated cells.

## ***1.2.Liver-Specific Gene Expression-The Example of Heme Oxygenase (HO)***

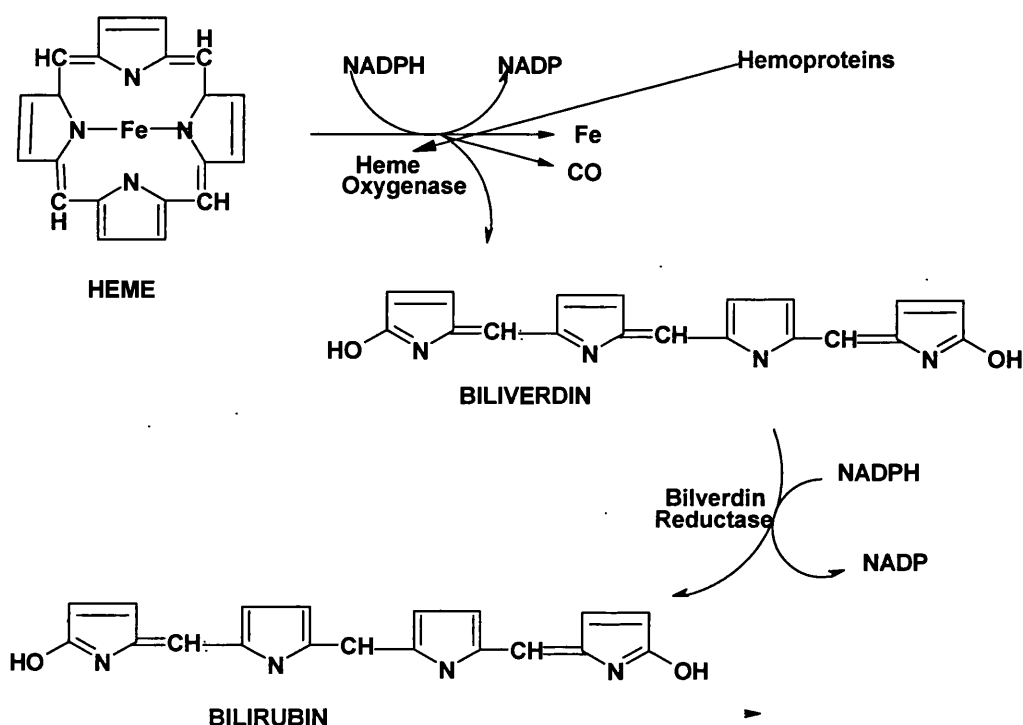
### **1.2.1. Definition and Structure of Heme Oxygenase (HO) [E.C.1.14.99.3]**

The expression of HO-1 is known to be particularly high in the liver (Maines, 1988). In 1968, Tenhunen *et al.* described the enzymatic conversion of heme to bilirubin by heme oxygenase (HO) using microsome fractions from various tissues, including the liver and spleen, it is now clear that the enzyme activity initially detected in the liver. Heme oxygenases are microsomal isozymes that catalyze the first and rate-limiting step in the degradation of heme. To date, three isoforms have been identified i.e. heme oxygenase-1 (HO-1), heme oxygenase-2 (HO-2) and heme oxygenase-3 (HO-3) (Maines *et al.*, 1986; McCoubrey *et al.*, 1997).

HO-1 is a 32-kDa protein that is normally synthesized predominantly on the free polysomes and then translocated to the endoplasmic reticulum where its carboxyl terminal is inserted into the membrane and its amino terminal protrudes into the cytoplasm (Shibahara *et al.*, 1980). HO-2 is a 36-kDa protein also located in the membrane of the endoplasmic reticulum (McCoubrey *et al.*, 1993) existing primarily in the brain and testes. Under physiological conditions, HO activity is the highest in the spleen where senescent erythrocytes are sequestered and destroyed, but its activity has also been observed in all systemic organs.

The HO-1 and HO-2 enzymes catalyze the cleavage of heme in presence of NADPH cytochrome P-450, to yield equimolar quantities of biliverdin IXa, carbon monoxide (CO) and iron as shown in **Figure 1.11**. (Maines, 1988;

Tenhunen *et al.*, 1968). Biliverdin in presence of biliverdin reductase is converted to bilirubin. It should be noted that the 33-kDa HO-3 isozyme also catalyses heme degradation although with a lower ability to oxidize heme than HO-1 (McCoubrey *et al.*, 1997; Quan *et al.*, 2001).



**Figure 1.11.** Schematic presentation of heme breakdown by heme-oxygenase.

The active site of the HO isozymes is believed to be located on the cytoplasmic site of the endoplasmic reticulum (Hino *et al.*, 1979). The catalytic site in HO-1 and HO-2 shows 100% homology, although overall their amino acid homology is only 58% (Maines, 1988). HO-1 is believed to be inducible whereas HO-2 is non-inducible. Although HO-1 and HO-2 enzymes have similar function in the cells, their size and structure are quite different. Furthermore these 2 isozymes

are immunologically different as shown by the use of HO-1 anti serum (Maines *et al.*, 1986). Thus the two isozymes are thought to be genetically different (Curse *et al.*, 1988).

Some of the main differences between HO-1 and HO-2 are summarized in **Table 1.1.**

**Table1.1.** The physical properties of HO-1 and HO-2.

<b>Property</b>	<b>HO-1</b>	<b>HO-2</b>
<b>Apparent monomeric molecular weight on SDS gel</b>	32,000	36,000
<b>Heat sensitivity (loss of activity at 60°C,10 min)</b>	30%	75%
<b>Ammonium sulfate precipitation</b>	0—35% saturation	35—60% saturation
<b>Electrophoretic behavior under nondenaturing conditions.</b>	Lower mobility	Higher mobility
<b>Requirement for activity</b>	O <sub>2</sub> , NADPH, NADPH <sub>-cyt</sub> P-450 reductase	O <sub>2</sub> , NADPH, NADPH <sub>-cyt</sub> P-450 reductase
<b>K<sub>m</sub> for hematin</b>	0.24μM	0.40μM
<b>Effect of heme ligands (CO, KCN, and NaN<sub>3</sub>)</b>	Inhibition	Inhibition
<b>Inducers</b>	Heme, bromobenzene, metal ions, phenylhydrazine, oxidizing agents	Not induced



<b>Product of heme (Fe-protoporphyrinIX) degradation</b>	Biliverdin IX	Biliverdin IX
<b>Substrate</b>	Hematin, Fe-hematoporphyrin, Fe-hematoporphyrin acetate, hemoglobin, cyt P-450 <i>b</i> , cyt P-420 <i>b</i> , cyt P-420 <i>c</i> .	Hematin, Fe-hematoporphyrin, Fe-hematoporphyrin acetate, hemoglobin, cyt P-450 <i>b</i> , cyt P-420 <i>b</i> , cyt P-420 <i>c</i>
<b>Specific activity</b>	4000 nmol of bilirubin/mg protein/hour	250 nmol of bilirubin/mg protein/hour
<b>Site of interaction with heme molecule</b>	Histidine residue at position -132 in human HO-1	Histidine residue at position -152 in human HO-2

### 1.2.2. Inducers of the HO-1 Gene

Although heme is the typical HO-1 inducer, several studies demonstrated that HO enzyme activity could also be stimulated by a variety of non-heme products. Stimulation of HO-1 expression is regulated primarily at the level of gene transcription (Choi and Alam, 1996). Indeed the rate of transcription of the HO

gene could be enhanced not only by heme but also by a variety of agents including oxidants such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), ultraviolet A (UVA, 320-380nm) radiation, heavy metals, sodium arsenite (NaAs<sub>2</sub>O<sub>3</sub>), thiol scavengers (i.e. Glutathione depletion), prostaglandins, endotoxin, cytokines, nitric oxide, phorbol esters and 12-O-tetradecanoyl-phorbol-13-acetate (Keyse and Tyrrell, 1989; Tyrrell *et al.*, 1993; Lavrosky *et al.*, 1993a; Maines, 1988; lu *et al.*, 1997; Lautier *et al.*, 1992; Elbirt *et al.*, 1998; Oguro *et al.*, 1996; Alam and Den, 1992; Rossi and Santoro, 1995, Carraway *et al.*, 1998, Terry *et al.*, 1998; Doi *et al.*, 1999 and Maines *et al.*, 1986). Also CdCl<sub>2</sub> has been shown to be necessary and sufficient for the HO-1 gene activation (Alam *et al.*, 2000).

In addition HO-1 is induced by drugs, metaloporphyrins, heat shock, shear stress, hematin promobenzene and phenyl hydrazine (Wagner *et al.*, 1997; Otterbein and Choi, 2000b). However, it should be noted that the induction of HO-1 by heat shock depends on the cell type. For instance a heat shock produces a great increase of HO-1 mRNA in Hep 3B cell line whereas it produces small increase in human fibroblast and HepG2 cell line with no increase in Hela cell line, macrophages and erythroblastic cells (lu *et al.*, 1997).

Shan *et al.*, (2000) demonstrate that the metallo-porphyrins especially Fe-protoporphyrin (heme) and Co-protoporphyrin (cobalt) strongly induce the activity of HO-1, and the key elements required for the metaloporphyrins-mediated induction of HO-1 are located at region -3.6 to -5.6 kb upstream of the transcription starting point due to the presence of the consensus AP-1 binding element at regions -3647 and -4576.

#### 1.2.2.1. The Significance of HO-1 Induction

Because of the growing list of HO-1 inducers, much effort has been concentrated on defining a unifying mechanism to explain the induction of HO-1 by chemically and structurally diverse agents. In 1991, Tyrrell and coworkers noted that many, if not all, HO-1 inducers could promote a cellular pro-oxidant state and that induction of HO-1 represented a general response to oxidant stress (Applegate *et al.*, 1991). The authors proposed that cellular oxidative stress, resulting from either increased production of reactive oxygen species or decreased levels of intracellular reductants, should be the common effector system for the various HO-1 inducers and that the induction of HO-1 might reflect a powerful mechanism by which the pro-oxidant state of the cells can be transiently lowered in order to avoid damage during a sustained oxidative stress. Subsequent studies by multiple investigators have confirmed the protective function of HO-1 induction and activity during injury initiated by both heme and non-heme insults. In the light of such studies, it was assumed that the cellular disruption by non-heme toxicants should also promote the release of, or access to, sufficient amounts of substrate for the induction of the HO-1 enzyme (Choi and Alam, 1996).

Although HO-1 is still known to catalyze only one enzymatic reaction (i.e. the oxidative cleavage of heme) new investigations in the field suggest that the cellular and physiological consequences of this reaction are considerably more significant since HO-1 appears to function as a cytoprotective molecule. Indeed, because of this reaction, HO-1 manifests antioxidant, anti-inflammatory and anti-apoptotic activities, modulates inter- and intracellular signaling mechanisms, and ultimately participates in the more general process of cellular homeostasis in response to injury (Alam, 2002).

The mechanism(s) by which HO-1 can mediate these cytoprotective functions is not clear. However the three major catalytic by-products, CO, ferritin, and bilirubin may represent potential targets:

- **Carbon monoxide, CO**, is unquestionably dangerous for the organism, as it can bind avidly to hemoglobin and replace oxygen (O<sub>2</sub>), can induce general hypoxia, increase the stability of oxyhemoglobin by shifting the dissociation curve to the left and impede O<sub>2</sub> delivery to the tissues. Furthermore, the consequent impaired dissociation of carbon dioxide (CO<sub>2</sub>) can lead to an increase in blood CO<sub>2</sub> levels and to the removal of the reflex stimulus to the respiratory centers in the brain (Otterbein and Choi, 2000b).

At high concentrations CO is lethal but recent studies in the field of neuro- and vascular biology consistently support the idea that CO at low concentrations exerts distinctly different effects on physiological and cellular functions (Mancuso *et al.*, 1997; Motterlini *et al.*, 1998; Verma *et al.*, 1993). To this regard it has been shown that CO generated from HO, the primary if not exclusive source of CO generation in the body, can regulate vasomotor tone (i.e. as a vasorelaxator) as well as neurotransmission (Snyder *et al.*, 1998; Morita and Kourembanas, 1995). A recent study from Nadel and coworkers (1998) clearly demonstrates that such effects of CO are mediated through the activation of guanylyl cyclase on the binding of CO to the heme moiety of this enzyme and subsequent cyclic guanine monophosphate (cGMP) generation (Cardell *et al.*, 1998). It is thought that CO may also possess anti-inflammatory effects such as the capacity to inhibit platelet activation or aggregation through activation of guanylyl cyclase and the subsequent generation of cGMP. Moreover, in models of lung injury,

administration of very low concentrations of exogenous CO to rats and mice had protective effects (Otterbein *et al.*, 1995 and 2000a). Changes in CO measurements in exhaled breath have been recently suggested as a direct indicative of increased HO-1 activity and cellular stress and therefore as a direct diagnostic marker of the severity of some of the disease processes such as asthma and diabetes (Paredi *et al.*, 1999; Yamara *et al.*, 1999).

- **Bilirubin** is best known as potentially toxic agent that accumulates in the serum of neonates, causing jaundice. However this byproduct of HO, which was originally considered as only a waste product of enzymatic cleavage of heme to be eliminated, is now considered as the most abundant endogenous antioxidant in mammalian tissues. Ames and coworkers (1987) were among the first investigators to discover that both biliverdin and bilirubin possess potent antioxidant activities such as scavenging peroxy radicals as efficiently as vitamin E (Stocker *et al.*, 1987; Stocker *et al.*, 1990). Rice-Evans and coworkers (1994) further demonstrated that bilirubin account for the majority of the antioxidant activity of human serum (Gopinathan *et al.*, 1994). HO-derived bilirubin has also been shown to be protective against both postischemic myocardial dysfunction (Clark *et al.*, 2000) and H<sub>2</sub>O<sub>2</sub>-mediated injury to neurons (Dore *et al.*, 1999). In a recent report, Vachharajani *et al.*, (2000) have shown that administration of biliverdin to rats modulates lipopolysaccharide-induced P- and E-selectin expression in the vascular system, providing evidence that bilirubin is able to modulate this inflammatory response regardless of the influences of HO-1, CO and/or ferritin. The authors further demonstrated that biliverdin administration also provides protection in a rat model of ischemic heart injury.

- **Ferritin** is a major iron storage protein in the cells. The release of potentially harmful free iron capable of generating hydroxyl radical through Fenton chemistry with the superoxide radical is rapidly sequestered into ferritin. This is thought to lower the pro-oxidant state of the cell by removing the free iron (Balla *et al.*, 1992). In (1993), Vile and Tyrrell showed that ferritin levels increase in the presence of UVA-mediated oxidative stress in human skin cells. The authors further demonstrated that HO-1-dependent release of iron also results in the upregulation of ferritin, which might provide protection after irradiation. Eisenstein *et al.*, (1991) clearly showed that ferritin is increased in tandem with HO-1 and decreased with inhibition in HO-activity. Balla *et al.*, (1992) also showed that induction of ferritin was cytoprotective in a model of oxidant stress, demonstrating that cytotoxicity was greatly reduced and occurred independently of HO-1 activity. Finally, Otterbein *et al.* (1997) in a model of endotoxic shock, demonstrated that when iron is chelated by the exogenous iron chelator desferrioxamine, no ferritin is induced and protection is ablated. From these studies a picture emerged showing that the HO-mediated iron release plays a role in increasing the intracellular level of ferritin which in turn protect the cells against further oxidative injuries.

### 1.2.3. Other Functions of HO-1

Although the by-products of heme cleavage play a significant role in the cytoprotective role attributed to HO-1, it is now more and more clear that the induction/ activity of this enzyme results in profound changes in cells' abilities to

protect themselves against oxidative injury. As mentioned previously, HO-1 has been shown to have anti-inflammatory, antiapoptotic and antiproliferative effects. Furthermore this enzyme appears to have salutary effects in diseases as diverse as atherosclerosis and sepsis (Morse and Choi, 2002).

Observations made in HO-1-deficient [HO-1(-/-)] mouse and human have highlighted the important metabolic and cytoprotective roles of this gene. For example Poss and Tonegawa (1997a and 1997b) generated HO-1(-/-) mice by targeted deletion of the mouse HO-1 gene. The mice exhibited incapacity to modulate body iron stores properly and were less resistant to hepatic injury by iron, indicating that HO-1 plays an important role in iron utilization. The authors reported that the majority of these HO-1(-/-) do not survive to term, and the mice that do survive to adulthood are abnormal and die within one year of birth.

A recent report (Yachie *et al.*, 1999) demonstrating the first identified case of a HO-1-deficient human patient lends additional support to the evolving paradigm that HO-1 serves to provide cytoprotection against oxidative stress. This patient exhibited phenotypic alterations as those observed in the HO-1(-/-) mice, including growth retardation, anemia, leukocytosis and increased sensitivity to oxidative stress.

The critical importance of HO is also demonstrated by reports (Lee, 1995; Muramoto *et al.*, 1999) that HO expression is conserved evolutionarily in prokaryotic bacteria as well as in plants and fungi. This conservation suggests that HO may play a role in diverse species as a modulator of cellular homeostasis, serving not only to degrade heme but also, via one or more of its catabolic by-products, to regulate a variety of critical cellular processes. For example, in plants, it is believed that HO possesses dual functions, one that generates tetrapyrroles and

another that recycles heme and chlorophylls, both being important after damage to the photoreactive center (Muramoto *et al.*, 1999). In most bacteria, iron is required for survival and it is particularly essential for pathogens to cause disease. To circumvent the low concentration of free extracellular iron, pathogenic bacteria have developed sophisticated mechanisms by which to acquire iron from iron-containing proteins found in their hosts. One such mechanism exploits a bacterial heme degradation enzyme similar to HO (Lee, 1995).

HO-1 plays also a crucial role in cardio-protection as it can partially rescue the heart from the ischemia/reperfusion injury by functioning both as an intracellular antioxidant and inducer of its own expression under stressful conditions such as preconditioning, that provokes oxidative stress and induces a large variety of oxidative stress-inducible gene (Yoshida *et al.*, 2001).

In addition expression of HO-1 is essential to prevent graft rejection presumably due to the generation of CO that prevents thrombosis and infraction of graft rejection (Sato *et al.*, 2001).

Furthermore, HO is believed to be involved in the regulation of vascular tone and blood pressure (Sabaawy *et al.*, 2001). And finally with regard to heme degradation, the activity of the enzyme seemed to be higher in tissues involved in the breakdown of red cells e.g.: spleen, bone marrow, and liver (Tenhunen *et al.*, 1970; Shibahara *et al.*, 1978).

#### **1.2.4. Inhibitors of HO**

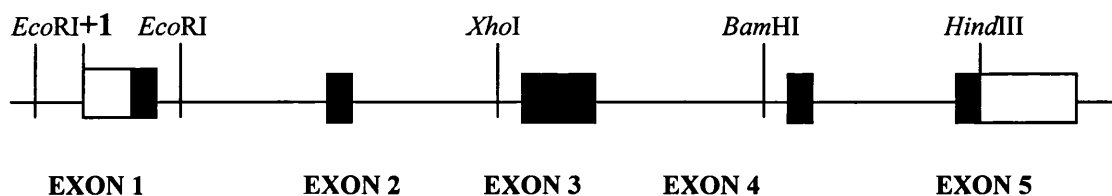
Some substances, which inhibit HO, include CO, KCN, NaN<sub>3</sub>, (Levere *et al.*, 1990; Sacerdoti *et al.*, 1989; Martasek *et al.*, 1991), and certain non-physiological metalloporphyrins such as Tinprotoporphyrin (Kappas *et al.*, 1984),



Znprotoporphyrin (Maines, 1981), and Cr- and Mn- protoporphyrin (Kappas *et al.*, 1984), inhibit the heme degradation activity by interacting with the heme binding site of HO protein.

### 1.2.5. Mouse Heme Oxygenase-1 Gene

The mouse HO-1 gene is approximately 7 kb in length, is organized into five exons interrupted by four introns as shown in **Figure 1.12**. The deduced amino acid sequence of the mouse HO-1 gene exhibits 93.4%, 82.3%, and 64.4% identity with the rat (Benton *et al.*, 1977), human (Yoshida *et al.*, 1988), and chicken (Evans *et al.*, 1991) proteins, respectively. Simultaneous alignment of all four sequences using the algorithm of Higgins and Sharp (1988) showed 57.4 % sequence identity. In addition, the histidine residue (at positions 25 and 132 of the rat sequence) proposed (Evans *et al.*, 1991; Ishikawa *et al.*, 1992) to bind the substrate heme to the active center of the enzyme is conserved in the mouse protein. The transcription initiation site was identified by S1 endonuclease mapping (S1) and primer extension (PE) analyses represented at position +1 (Alam *et al.*, 1994).



**Figure 1.12.** Partial restriction map and structural organization of the mouse HO-1 gene, the position of the exons are marked by open boxes (untranslating region) and solid boxes (protein coding region).

#### 1.2.5.1. Mouse Heme Oxygenase-1 Upstream Promoter Elements

In 1992, Alam *et al.* demonstrated that the basal expression of a chimeric gene consisting of approximately 7 kb of the 5'-flanking region of the mouse HO-1 gene fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene was two to ten fold greater of an analogous construct containing only 1287 bp of the 5'-flanking region in transiently transfected cultured cells. The authors localized this enhancer activity to a 268 bp fragment positioned approximately 4 kb upstream of the transcription initiation site. Using the Dnase I protection assay, they further demonstrated that this fragment called "SX2" contained two high affinity protein binding sites i.e. region A (TGAGTCA) and region B (TGTGTCA) that resembled the consensus binding site, TGA(G/C)TCA, of the activator protein-1 (AP-1) family of transcriptions factors. Further functional studies revealed that indeed the SX2 region, which mediates basal level and inducer-dependent activation of mouse HO-1 gene, contains two functional AP-1 binding sites (i.e. AP-1a and AP-1b) and that mutation of both of these elements diminishes (by 50-70%), but does not abolish the enhancer activity, suggesting that other sequences should contribute to enhancer function of the 5'-flanking region of the mouse HO-1 gene. In (1994), Alam showed that directly upstream of these AP-1 binding sites are two copies of a sequence motif, which resembles elements found in cellular and viral genes that are known to interact with the C/EBP protein family of transcription factors. These SX2 sequences bind specifically to liver-enriched, heat-stable nuclear proteins and confer C/EBP $\alpha$ -dependent transactivation of the heterologous CAT gene. Further mutational analyses indicated that both the AP-1 and C/EBP binding elements are required for optimal activation of the mouse HO-1 gene by cadmium and other metals. Since multiple copies of the AP-1 binding elements, but not C/EBP

elements, are necessary and sufficient for activation of a heterologous gene by CdCl<sub>2</sub>, the AP-1 proteins appeared to play the primary role in the activation process (Alam, 1994). The same year Shibahara and coworkers (Takeda *et al.*, 1994) isolated the human homolog of the SX2 region and in contrast to the mouse studies by Alam (1994), their studies indicated that the AP-1 elements do not mediate induction of a heterologous gene in response to cadmium.

Using transient expression assays with CAT reporter gene and deletion analysis within the 5'-flanking region of the mouse HO-1 gene, Alam *et al.*, (1994) further demonstrated that the basal promoter activity in several cell lines (i.e. fibroblast L929, rat C6 glioma and rat hepatoma cells) is located within 149 bp of the upstream sequence. This proximal promoter region of the mouse HO-1 gene contains several sequence elements that are not only conserved in both the rat and human HO-1 genes but also resembles consensus binding sites of various transcription factors including AP-1, AP-4, C/EBP and c-Myc:Max/USF.

The DNA sequences upstream of the transcription initiation site of the mouse and rat HO-1 genes (Muller *et al.*, 1987) are highly conserved, with the notable exception of an insertion of polypyrimidine tract, comprised mainly of the repeated pentamer TCTCT, in the mouse gene. These homopurine/homopyrimidine polymorphic sequences, possessing Z-conformation potential, have been shown to negatively affect transcriptional activity of genes such as the rat prolactin (Naylor and Clark, 1990). These alternating repeats are present in the promoter regions of several genes including the human c-Ki-ras proto-oncogene (Hoffman *et al.*, 1990) and the human HO-1 gene (Kimpura *et al.*, 1997). Excluding the polypyrimidine tract, the mouse and rat HO-1 gene 5'-fanking regions exhibit 86% sequence similarity (Alam *et al.*, 1994).

The mouse HO-1 gene 5'-flanking region also contains three heat shock elements (HSEs), one of which is analogous to the rat HSE motif, called HSE $\alpha$  (Muller *et al.*, 1987; Alam *et al.*, 1994). However, none of these putative HSEs, contain the three intact GAA/TTC blocks, which may explain the weak response of the mouse HO-1 to hyperthermia (Alam *et al.*, 1989). On the other hand, the human HO-1 gene promoter contains such intact HSE, but is unresponsive or only minimally responsive to hyperthermia in several cell lines (Shibahara *et al.*, 1989; Mitani *et al.*, 1990).

Heavy metals activate HO-1 gene transcription and the rat gene contains a putative metal regulatory element, MRE (Muller *et al.*, 1987), that is completely conserved in the mouse gene. However, transient expression analyses performed by Alam *et al.*, (1994), indicated that this sequence containing core heptanucleotide 'TGCACTC' identical to that of the strongest MRE of the mouse metallothionein gene was not responsive to cadmium or zinc. To identify the specific regions within the 5'-distal sequence of mouse HO-1 gene responsible for its induction by both heme and heavy metals, the authors stably transfected several constructs containing either the entire mouse HO-1 gene or various portions of the 5'-flanking region into rat C6 glioma cells. The simultaneous and quantitative analysis of the mouse and rat HO-1 mRNAs indicated that the distal 5'-sequences, between positions -3.5 and -12.5 kb, were required for induction of mouse HO-1 gene transcription.

To this regard, in (1995), Alam *et al.*, described a second regulatory region called, AB1 (161 bp), located approximately 10 kb upstream of the mouse HO-1 gene with properties similar to SX2. The authors further demonstrated that this fragment contains three copies of the AP-1 binding sequence and mutation of these elements

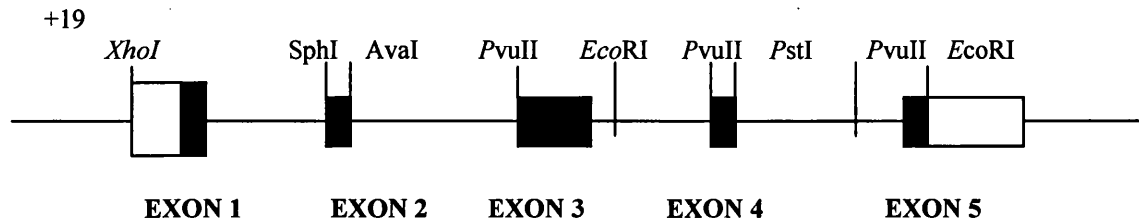
abolishes induction not only by CdCl<sub>2</sub>, but also by heme and a variety of other HO-1 inducers. These results demonstrated for the first time that the mechanism of HO-1 gene activation by diverse agents converges at the same DNA motif and provided further evidence for the importance of Fos/Jun proteins in this process. The signaling pathway leading to the expression and/or activation of the transcription factors, however appeared to be inducer-dependent. For example Inamdar *et al.*, (1996) showed that the AP-1 binding sequence, TGAGTCA, is necessary but insufficient for gene activation by heme or cadmium. Furthermore the authors reported that the five AP-1 motifs of the SX2 and AB1 enhancers exhibit sequence similarity beyond the AP-1 heptad and that the heme response element (HRE) is actually an extended AP-1 binding sequence, i.e. (T/C)GCTGAGTCA. This motif resembles the binding site of the product of the v-Maf oncogene (Kataoka *et al.*, 1994) and of the transcription factor nuclear factor-erythroid 2, NF-E2 (Andrews *et al.*, 1993a; Andrews *et al.*, 1993b). The HRE is in fact a composite element that also contains overlapping sequences for the antioxidant responsive element, ARE, i.e. GCnnnGTCA (Rushmore *et al.*, 1991) and the AP-1 heptad. The ARE is a transcriptional regulatory element that plays a role in the expression of phase II enzymes such as Glutathione-S-transferase and NAD(P)H:quinone oxidoreductase (Rushmore *et al.*, 1991; Xie *et al.*, 1995; Wasserman and Fahl, 1997).

The composite HRE/AP-1/ARE *cis*-acting elements present in several copies within the mouse HO-1 enhancer regions are now generally termed as the stress response elements, StREs (Choi and Alam, 1996) that are essential for inducer-dependent gene activation. The consensus StRE is defined as (T/C)GCTGAGTCA, and as mentioned above, it does resemble the consensus binding site, TGA(C/G)TCA, for the AP-1 class of transcription factors that have been proposed to be responsible for

HO-1 gene activation (Alam and Den, 1992; Alam J., 1994). These predictions were based, among other reasons, on the observations that AP-1 proteins bind to individual StREs, that the DNA binding of c-Jun.c-Fos heterodimer is subjected to redox regulation, and that expression and activities of some members of the Jun and Fos family of proteins are stimulated by many of the same agents that induce HO-1 expression (Alam *et al.*, 1999). A role for AP-1 proteins in HO-1 gene regulation is further supported by recent studies demonstrating that pharmacological inhibition of AP-1 activity attenuates interleukin-1 $\alpha$  or tumor necrosis factor- $\alpha$ -mediated induction of HO-1 mRNA levels in human endothelial cells (Terry *et al.*, 1998) and ectopic expression of a dominant-negative mutant of c-Jun inhibits arsenite-mediated activation of the chicken HO-1 promoter in hepatoma cells (Elbirt *et al.*, 1998). Also, in (1995), Camhi *et al.*, showed that lipopolysaccharide (LPS)-induced HO-1 gene transcription in RAW264.7 macrophages is mediated by the distal enhancer SX2. Further deletion analyses from the same laboratory demonstrated that the transcription factor AP-1 is critical for AB1-mediated HO-1 gene activation by LPS (Camhi *et al.*, 1998). Their data suggested that LPS regulates HO-1 gene transcription in part by inducing the production of reactive oxygen species, which initiate signal transduction pathway(s) leading to the activation of AP-1-dependent HO-1 gene transcription.

#### **1.2.6. Human Heme Oxygenase-1 Gene**

The human HO-1 gene coding sequence is about 14 kb long and organized into five exons and four introns as shown in **Figure 1.13**.



**Figure 1.13.** Restriction map and structural organization of the human HO-1 gene, the position of the exons are marked by open boxes (untranslating region) and solid boxes (protein coding region).

The transcription initiation site was identified by S1 nuclease mapping and primer-extension analysis. The size of the major primer cDNA was about 80 nucleotides indicating the transcription initiation site located upstream from the *HpaII* site. The S1 probe located from position -99 to +70 labeled at the *BanII* site as shown in **Figure 1.14.** (Shibahara *et al.*, 1989).

```

-180  ATGGCCAGACTTTGTTTCCCAGGGTCATATGACTGCTCCTCTCCACCCACACTGGCCC
-120  GGGGCGGGCTGGGCGCGGGCCTGCGGGTGTGCAACGCCGGCCAGAAAGTGGGCATCA
      BanII
-60   GCTGTTCCGCCTGGCCCACGTGACCCGCCGAGCATAAAATGTGACCGGCCGCGGCTCCGGC
1     *AGTCAACGCCTGCCTCCTCTCGAGCGTCCTCAGCGCAGCCGCCGCCCGCGGAGCCAGCA
      |-----XhoI-----Exon 1-----|
61    GAACGAGGCCAGCACCGGCGATGGAGCGTCCGCAACCCGACAGGCAAGCGCGGGGC
      BanII      HpaII                               Intron 1

```

**Figure 1.14.** Nucleotide sequence of the 5'-flanking region and exon 1 of the human HO-1 gene adapted from Shibahara *et al.*, (1989). The transcription initiation site is marked with an asterisk. A TATA-like sequence is indicated by a thin overline. The ATG codon for initiating methionine is indicated by a thick overline. Thin underlines indicate the nucleotide residues which are aligned to the same nucleotides of the rat HO gene (Muller *et al.*, 1987).

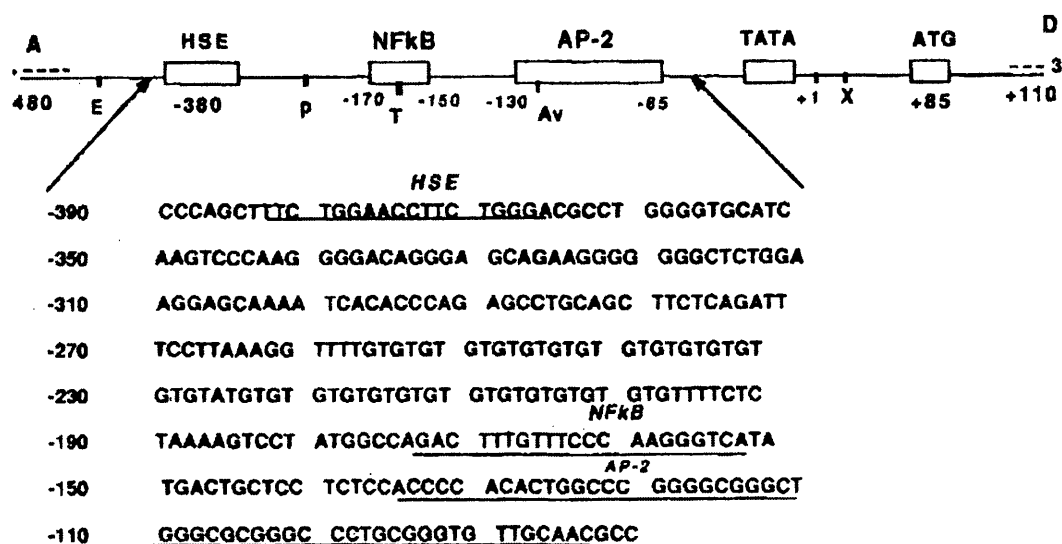
#### 1.2.6.1. Human Heme Oxygenase-1 Upstream Promoter Elements

The upstream element of the HO promoter is essential for constitutive activity of the HO promoter (Sato *et al.*, 1990). The *cis*-acting element within 120 bp of the mRNA cap site is involved in the inducible stimulation of transcription by a variety of agents including oxidants (Tyrrell *et al.*, 1993).



One of the mechanisms by which hormones, growth factors, and other stimuli induce the expression of genes is by activating various transcription factors. These processes may be part of the mechanism by which the agents, including heme, increases HO expression and activity (Quan, *et al.*, 2002).

Using DNase I foot print assays, Lavrovsky *et al.* (1994) demonstrated the presence of NF- $\kappa$ B, HSE and AP-2 binding sites in the proximal part of the promoter region of the human HO-1 as shown in **Figure 1.15**.



**Figure 1.15.** Schematic representation of the human HO-1 proximal promoter region containing the HSE, NF- $\kappa$ B and AP-2 binding sites and the ATG initiation codon (adapted from Lavrovsky *et al.*, 1994)

The authors observed that the most pronounced effect of heme is on NF- $\kappa$ B and AP-2 binding activities and since the activation of NF- $\kappa$ B and AP-2 is often associated with the immediate response of the cell to an injury, they suggested that the presence of these regulatory sequences upstream of HO-1 is an strong indicator of the defense

mechanism activity of HO-1 against tissue injury (Lavrovsky *et al.*, 1994). A brief description of these transcription factor binding sites is provided below:

- AP2 is a transcription factor binding site that has been identified in the enhancer regions of viral and cellular genes, including simian virus SV40, human metallothionein IIA, and human T-cell leukemia virus type I (Haslinger *et al.*, 1985; Williams *et al.*, 1988). Phorbol esters, cAMP and retinoic acid can induce the AP-2 activity, whose activation is associated with the immediate response of the cell to an injury (Imagawa *et al.*, 1987; Snape *et al.*, 1991). Using the computer analysis, Lavrovsky *et al.*, (1993a) indicated the presence of two AP-2 like regulatory site in the upstream region, one having the binding site from position “-116 to -124” with the consensus sequence GCCCGGGGC and the second one from position “-7 to -15” with the sequence of GGCCGCGGC. The authors used the Dnase I footprint assay to confirm the presence of AP-2 binding sites within the human HO-1 promoter. It appears that these binding site play an important role in the induction of HO gene during inflammatory and oxidation stress.
  
- NF- $\kappa$ B is a transcription factor that is activated in many different cell types by a challenge from primary and secondary pathogenic stimuli. This activation leads to a rapid induction of genes encoding defense and signaling proteins (Nolan *et al.*, 1993). NF- $\kappa$ B contains two binding sites from which one is transcriptional site AAGTCCCAAG “-370 to -379bp” as found to share the same site with the heat shock responsive element (HSE) at the 5' flanking region (Lavrovsky *et al.*, 1993a; Faisst *et al.*, 1992; Shibahara *et al.*, 1989). The second one was found at position “-156 to -166” bp and its

sequence is GCTCCTCTCCA. The Dnase I footprint assay confirmed the presence of these NF- $\kappa$ B binding sites within the human HO-1 promoter (Lavrovsky *et al.*, 1993a). Carbon monoxide (CO) generated by HO-1 during heme catabolism is also dependent on the activation of NF- $\kappa$ B transcription factor in endothelial cells. This is thought to protect the endothelial cells from undergoing tumor necrosis factor- $\alpha$ -mediated apoptosis by exerting an anti-apoptotic effect (Brouard *et al.*, 2002).

In addition to these *cis*-regulatory elements within the 5'-flanking region of the human HO-1 gene, Sato *et al.*, (1990) have identified the upstream stimulatory factor (USF) binding site as CACGTGACCCG located 34 bp upstream from the transcription initiation site of the HO-1 gene. The authors provided data suggesting that USF contributes to the transcription of the human HO-1 gene.

In 1994, Shibahara and coworkers (Takeda *et al.*, 1994) performed transient expression assays in four human cell lines including HeLa to analyze the cadmium mediated inducibility of the fusion genes containing the firefly luciferase gene as a reporter under the control of the human HO-1 gene promoter. Their results revealed that the region between 4.5 and 4 kb upstream from the transcriptional initiation site is responsible for cadmium-mediated inducibility on the fusion gene. Further functional analyses in HeLa allowed limiting the localization of the cadmium responsive element (Cd<sup>2+</sup>RE) to 20 bp. The bandshift assays demonstrated that this 20 bp element is specifically bound by nuclear protein(s) of HeLa cells although these binding activities were unchanged by the treatment with cadmium. Finally, using the synthetic oligonucleotides that had various base changes, the authors identified a 10 bp sequence, TGCTAGATTT, required for the cadmium inducibility

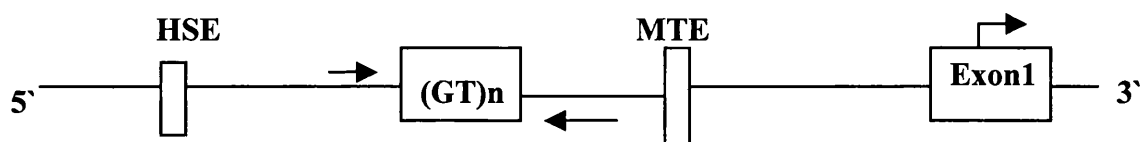
and *in vitro* protein binding. The following year, the same group (Takeda *et al.*, 1995) showed by transient expression assays that Cd<sup>+2</sup>RE of the human HO-1 gene is not responsive to zinc, whereas a metal regulatory element (MRE) of the human metallothionein IIA gene is able to respond to either cadmium or zinc. The consensus sequence of the Cd<sup>+2</sup>RE is different from that of the MREs of the mouse metallothionein I promoter, TGCRCNC (R, purine; N, any nucleotides) (Stuart *et al.*, 1985), except for the TGC trinucleotides as its 5'-end. Takeda *et al.* (1995) further demonstrated that the Cd<sup>+2</sup>RE is recognized by a certain nuclear proteins which is however unable to bind to an MRE of the metallothionein IIA gene, suggesting that the metal-selective activation of each gene promoter is mediated by a separate mechanism. This is in agreement with the notion that living organisms have acquired the two independent systems involved in detoxification of heavy metals during evolution.

In (1996), Lavrovsky *et al.*, examined several human HO-1 promoter-driven CAT constructs to analyse promoter activity of the human HO-1 gene in microvessel endothelial cells. The HO-promoter activity was upregulated by IL6 and downregulated by glucocorticoids. The reporter CAT assays revealed that the promoter region (56 bp) between -180 and -120 was responsible for upregulation by growth factors, as well as for glucocorticoid-directed downregulation. This region had a high homology to sequences in the human platelet glycoprotein IIb. The analysis of DNA-protein complexes revealed that the DNA fragment contains a sequence recognized by the STAT3/acute phase response factor.

The (GT)<sub>n</sub> dinucleotide repeat in the 5'flanking region of the human HO-1 gene shows length polymorphism and could modulate the level of gene transcription (Kimpapa *et al.*, 1997; Exner *et al.*, 2001). Recent evidence from the literature has

linked the large size of a (GT)<sub>n</sub> repeats present within the HO-1 gene promoter to reduced inducibility of the gene (Exner *et al.*, 2001; Yamada *et al.*, 2000; McGinnis *et al.*, 1995). For example the study from Yamada and coworkers (2000) suggest that the expression of the HO-1 gene could be altered according to the number of (GT)<sub>n</sub> repeats and that this microsatellite polymorphism may be associated with the development of oxidative stress-inducing diseases (Yamada *et al.*, 2000). Using a population of 100 smokers with or without the chronic pulmonary emphysema (CPE), the authors grouped the polymorphisms of the (GT)<sub>n</sub> repeat into three classes: class S allele (<25 repeats), class M allele (25-29 repeats ), and class L allele (≥ 30 repeats). They found that the basal luciferase activity was significantly higher in cells transfected with a (GT)<sub>20</sub> repeat than in those with either (GT)<sub>29</sub> (GT)<sub>38</sub>.

It is also suggested that the poly GT sequences present in the HO-1 gene promoter is highly polymorphic due to the location of the (GT)<sub>n</sub> repeat between the putative heat-shock elements (HSE) and the *cis*-regulatory element (MTE) as shown in **Figure 1.16.** (Okinaga *et al.*, 1996).



**Figure 1.16.** Structural organization of the (GT)<sub>n</sub> repeats in the human HO-1 gene.

In (1997), Lu *et al.*, reported a negative regulatory region in the human HO-1 gene. They demonstrated that the basal promoter activity of the luciferase reporter gene

which is regulated by the –1416 bp upstream region of the human HO-1 gene was lower than that by the –121 bp upstream region of the gene when both reporter constructs were transfected in primary cultures of chick embryo liver cells.

In (1999), Takahashi *et al.*, assessed approximately 4 kb of the 5'-flanking region of the human HO-1 gene for basal promoter activity and sequenced approximately 2 kb of the 5'-flanking region. Basal level expression of a series of deletion mutants of the 5'-flanking region linked to the luciferase was tested in two human cell lines of HepG2 (human hepatoma cells) and Hela (human cervical cancer cells). The authors found a positive regulatory region at position –1976 to –1655 bp that functioned in HepG2 cells but not in Hela cells. A negative regulatory region was also found at position –981 to –412 bp that functioned in both HepG2 cells and Hela cells.

To elucidate further the regulation of the human HO-1 gene expression and to gain insight into its tissue preferential expression, in the present study the 4.7 kb of the 5'-flanking region of the human HO-1 gene was examined for basal promoter activity in both HepG2 and Hela cells using a series of deletion mutant constructs linked to the luciferase gene.

## 1.3. Aim of the Work

To design new plasmid DNA expression vectors of liver-specific gene for the purpose of targeted gene delivery.

### 1.3.1. Objectives

1. The identification of liver-specific *cis*-acting regulatory elements.
2. Design of synthetic promoters/enhancers containing the candidate liver specific *cis*-acting elements.
3. The use of the above elements to drive the expression of luciferase gene in a EBV-based reporter vectors transiently transfected into both liver-specific HepG2 and non-liver HtTA-1 cell lines.
4. To analyze the *cis*-acting elements within the promoter of HO-1 gene that is known to be highly expressed in the liver.
5. To use EBV-based constructs containing 4.7 kb upstream 5'-flanking region of HO-1 gene in transient transfection assays within two cell lines of liver-specific HepG2 and non liver HtTA-1.

## **CHAPTER 2    Materials and Methods**

### ***A. Plasmid Constructions***

#### **A.1. Molecular Biology Materials**

All biochemicals were analytical grade and purchased from Sigma Chemicals, UK, the DNA ladder was obtained from (Fermentas MBI, UK), and all of the restriction enzymes were obtained from (GIBCO, UK) except where indicated.

##### **A.1.1. Antibiotics**

Ampicillin (sodium salt) was prepared as a 100 mg/ml solution in water and filter sterilized using a 0.2 µm pore membrane (Millipore, UK) and stored at –20°C for up to 3 months. Working concentration used was 100 µg/ml.

##### **A.1.2. Cell Resuspension Solution**

50 mM Tris-Hcl (pH 7.5), 10 mM EDTA, 100 µg RNase.

##### **A.1.3. Cell Lysis Solution**

0.2 M NaOH, 1% SDS.

##### **A.1.4. Neutralisation Solution**

1.32 M potassium acetate (pH 4.8).



#### **A.1.5. TE Buffer**

Ten mM Tris, 1 mM EDTA, (pH 8).

#### **A.1.6. Ethidium Bromide**

Ten mg/ml, 0.2 gm of ethidium bromide resuspended in 20 ml of H<sub>2</sub>O, and stored in dark.

#### **A.1.7. TAE Buffer**

0.04 mM Tris-Acetate, 0.001 mM EDTA.

#### **A.1.8. TBE Buffer (GIBCO, UK)**

10 X TBE buffer is a solution of 1 M Tris, 0.9 M boric acid, and 0.01 M EDTA.

#### **A.1.9. LB Agar**

Bacto-Tryptone	10 g/l
----------------	--------

Bacto-yeast extract	5 g/l
---------------------	-------

Sodium chloride	5 g/l
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Reagents for preparing LB were obtained from (Difco, UK). Solutions were sterilised by autoclaving at 121°C for 20 minutes.

#### **A.1.10. Lysis Buffer (Promega, UK)**

(1 x :25 mM Tris-phosphate, pH 7.8: 2 mM DTT; 1,2-diaminocyclohexane-N,N,N,N-tetraacetic acid; 10 % glycerol; 1 % Triton X-100 and BSA 1 mg/ml).

#### A.1.11. Preparation of Tfb I Buffer as in Table 2.1.

**Table 2.1:**

Component	Quantity	Final concentration
KCl	0.223 g	30 mM
RbCl	1.209 g	100 mM
CaCl <sub>2</sub>	0.110 g	10 mM
MnCl <sub>2</sub>	0.985 g	50 mM
(% V/V) Glycerol	15 ml	15 %
Water	To 100ml.	

Adjusted to pH 5.8 with 0.2 M acetic acid and filter-sterilized (0.2 µm).

#### A.1.12. Preparation of Tfb II Buffer as in Table 2.2.

**Table 2.2.**

Component	Quantity	Final concentration
MOPS	0.209 g	10 mM
CaCl <sub>2</sub>	0.831 g	75 mM
RbCl	0.121 g	10 mM
(% v/v) Glycerol	15 ml	15%
Water	To 100 ml	

Adjusted to pH 6.6 with 1 M KOH and filter-sterilized (0.2 µm).

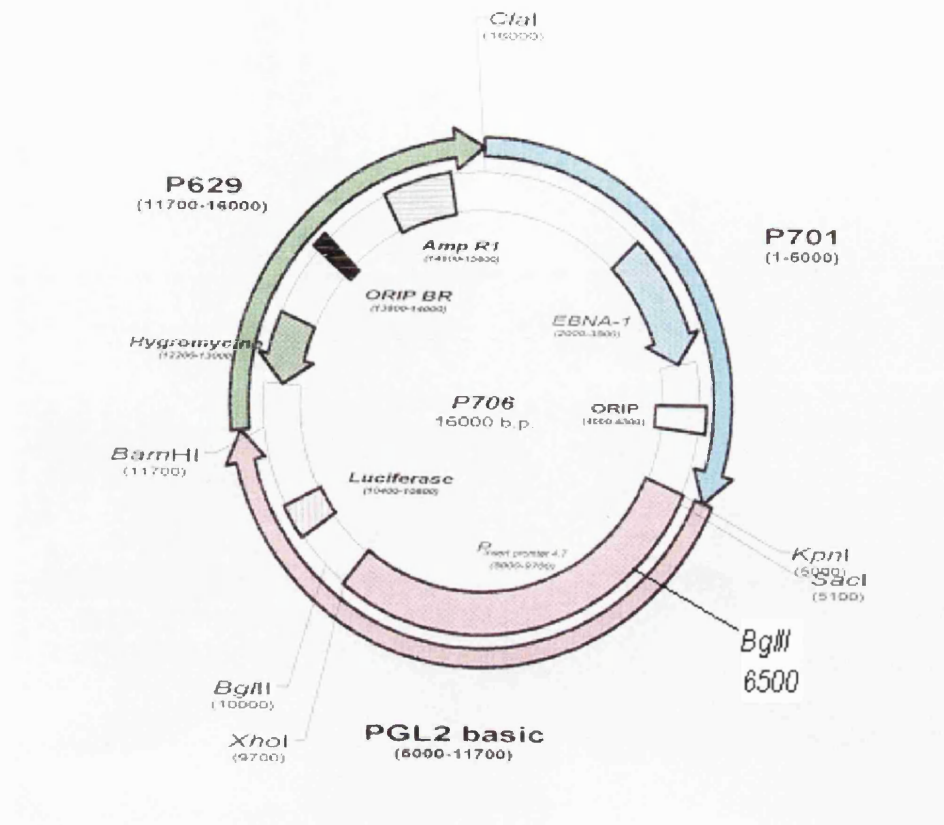
## 2.1. Molecular Biology Methods

### **p706/HO4.7 Plasmid**

The majority of the experiments described in this thesis were carried out either with the p706 /HO4.7 plasmid (16 kb) or its derivatives (see section 5.1.), all kind gifts from the laboratory of Professor R. M. Tyrrell (Pharmacy and Pharmacology Department).

In addition to these plasmids, several novel constructs were also made during this project based mostly on the modification of either the promoter or enhancer elements of the p706 plasmids (see section 5.2.).

The p706/HO4.7 contains the 4.7 kb of the proximal promoter region of the HO-1 and was originally constructed to identify the key elements of the HO-1 promoter responsible for the transcriptional activation of HO-1 by ultraviolet A radiation. p706/HO4.7 vector is constructed from a combination of three-linearised plasmids p701 *Clal/KpnI*, p629 *Clal/BamHI* and p653 *KpnI/BamHI*, as shown in **Map 1**.



**Map1: p706/HO4.7**

p706 itself is an Epstein Barr Virus (EBV-based episomal vector) derived from the basic episomal plasmid p629 [205MT (ID) poly CAT], originally obtained from M. R. James (Human Polymorphism study center, Paris). The *Clal/BamHI* and the *Clal/KpnI* fragments of p629 (called p701) were recombined with the *KpnI/BamHI* fragment of pGL2 basic (called p653) to generate p706.

(1) **p701 *ClaI/KpnI* fragment:** This fragment contains the latent origin of replication (oriP), and Epstein Barr Nuclear Antigen-1 (EBNA-1) sequences of the EBV-based vector p629.

(2) **p629 *ClaI/BamHI* fragment:** This fragment contains both hygromycin- and ampicillin-resistance genes of the EBV-based vector p629.

(3) **p653 *KpnI/BamHI* fragment:** This fragment contains the firefly luciferase insert and SV40pA sequences from the original pGL2-basic vector plus the 4.7 kb *XhoI/KpnI* fragment that is the 4.7 kb upstream region of the human HO-1 promoter inserted next to the luciferase insert within the multiple cloning site of the vector.

It is worthy of note that the pGL2-Basic vector lacks eukaryotic promoter and enhancer sequences thus allowing maximum flexibility in the cloning of putative regulatory sequences. Expression of luciferase activity in extracts of cells transfected with this plasmid depends on the insertion and proper orientation of a functional promoter upstream of the genetic luciferase “*luc*” element.

#### **2.1.1. Propagation, Isolation and Purification of p706/HO4.7**

The present project began by isolation and purification of p706/HO4.7 plasmid from an *Escherichia coli* (*E. coli*) strain “sure cut” bacterial strain (Promega, UK) that was originally transformed with this construct. For this purpose, the bacterial strain containing the p706/HO4.7 plasmid was cultured overnight on an Luria-Bertani (LB) agar plate (see section A.1.9) supplemented with 100 µg/ml ampicillin (see section A.1.1). After checking the controls, transfer single colony forming units from the LB agar plates into 10 ml of LB broth containing the appropriate antibiotic.

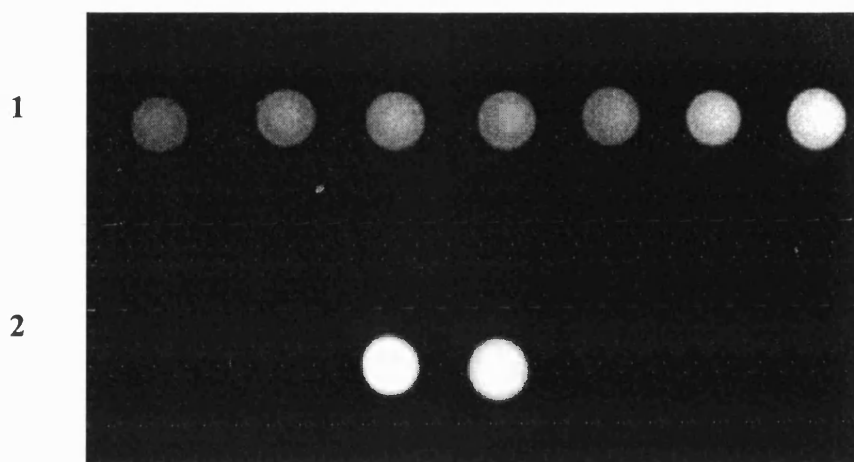
The cultures were grown overnight at 37°C on a shaking incubator at 300 rpm using the New Brunswick Scientific shaker (UK). The next day 5 ml portions of each culture were inoculated into 150 ml of LB broth containing the same antibiotic and this culture was grown overnight (i.e. 16-18 h) at 37°C on a shaking incubator. The next day, cell suspensions were centrifuged for 5 min at 14,000 rpm (10000 x g) in a microcentrifuge and the supernatant discarded. The pellets were resuspended in 250 µl of resuspension solution (see section A.1.2) by gentle vortexing. This was followed by the addition of 250 µl of cell lysis solution (see section A.1.3) to each suspension and the subsequent mixing by inversion in order to obtain a clear solution. The suspensions were neutralized with 350 µl of neutralization solution (see section A.1.4) and then centrifuged at 14000 rpm (10000 x g) for 10 min. The supernatants were then removed and transferred to a barrel minicolumn/syringe assembly containing the DNA purification resin (Promega kit, UK). A vacuum was then applied to pull the lysate solution into the minicolumn. Thereafter to each column, 700 µl of washing solution was added followed by the application of a vacuum to draw the solution through the minicolumn. The latter procedure was repeated a second time. The minicolumns were then transferred into a 1.5 ml microcentrifuge tube (Tyco Healthcare Group, MA, USA) and centrifuged at 14000 rpm (10000 x g) for 1 min to remove any possible residue. The minicolumns were then transferred into a clean 1.5 ml microcentrifuge tube. Finally, the isolated DNA was eluted from the column in TE buffer (see section A.1.5). This was incubated for 10 min at 60°C and then spin at 14,000 rpm for 1 min.

## **2.1.2. Methods for Quantification of p706/HO4.7 Plasmid DNA**

### **2.1.2.1. Ethidium Bromide Dot Analysis**

Pure DNA solutions were prepared as standards at a series of concentrations of 0, 1, 2.5, 5, 7.5, 10 and 20 µg/ml. To each 4 µl of the standard and the DNA solutions under test (e.g. purified plasmids), 4 µl of 1 µg/ml ethidium bromide (see section A.1.6 ) was added.

The contents of each tube were spotted into Saran plastic wrap (SOD, UK) covering a thin glass plate. The glass plate was then placed under a UV transilluminator and photographs were taken using a Polaroid MP4 camera and Polaroid 637 film. The fluorescence of the unknown was compared with the fluorescence of the different spots of the standard DNA to estimate the approximate concentration of the unknown DNA solutions see **Figure 2.1**.



**Figure 2.1. Estimation of the concentration of the DNA fragments by ethidium bromide dot analysis.** Lane 1 contains the DNA standards dots at concentrations of 0, 1, 2.5, 5, 7.5, 10 and 20  $\mu\text{g/ml}$  and lane 2 contains the unknown DNA samples dots.

#### **2.1.2.2. Spectrophotometer Quantification of p706/HO4.7 Plasmid DNA**

Accurate estimation of the concentration of the DNA was then performed using the “GeneQuant II spectrophotometer” (Milton Roy Spectronic) as follows: 100  $\mu\text{l}$  of  $\text{H}_2\text{O}$  was placed in a reference cuvette to adjust zero level at 320 nm; 2  $\mu\text{l}$  of this water was withdrawn and replaced with 2  $\mu\text{l}$  of the DNA solution to be tested. The absorbance was read at 320 nm. The concentration of the DNA of the test solution was also obtained using the absorbance at 260 nm ( $A_{260}$ ) and 280 nm ( $A_{280}$ ). The purity of DNA in the original solution was determined as follows:



$$\text{Ratio} = A_{260} / A_{280}.$$

Ratios between 1.8-2 were consistently obtained. The concentration of DNA was then calculated according to the following formula:

$$\mu\text{g/ml DNA} = \text{Dilution factor} \times 50 \times A_{260}$$

### **2.1.3. Preparation of the p706/*BglIII*-*KpnI* DNA Fragment**

**Summary:** To prepare a series of p706 constructs containing 1, 3 or 5 repeat of HNF-4 responsive elements, the *KpnI*- *BglIII* insert of p706/HO4.7 containing the 4.7 kb upstream promoter region of the human HO-1 was removed in two steps as follows:

The p706/HO4.7 plasmid was first digested with the *KpnI* restriction enzyme. An aliquot of the reaction mixture was loaded on a 1% agarose gel (in 1 x TAE buffer) (see section A.1.7) to monitor the level of linearization of the plasmid. Next, the reaction mixture containing the linearized p706/HO4.7 was purified by ethanol precipitation, and subjected to a second enzymatic digestion with the *BglIII* restriction enzyme. The digested material was then loaded on a 1% agarose gel (1 x TAE) and the p706/*KpnI*-*BglIII* fragment was extracted from the gel and further purified by ethanol precipitation.

Subsections 2.1.3.1 to 2.1.3.5. describe in further detail the preparation of the p706/*KpnI*-*BglIII* fragment.

### 2.1.3.1. Digestion of the Vector p706/HO4.7 with the *KpnI* Restriction Endonuclease

The digestion reaction contained the components shown in **Table 2.3**.

**Table 2.3.**

Reagent	Volume (μl)
DNA plasmid (p706/HO4.7 kb)	45 μl (= 20 μg)
<i>KpnI</i> enzyme	80 units
(10 x) REACT® 4 buffer	10 μl
Milli-Q H <sub>2</sub> O	37 μl
Total	100 μl

The above reaction was prepared in 5 replicates in microcentrifuge tubes and incubated overnight at 37°C. The next day, the samples were heated at 65°C for 10 min to stop the enzymatic digestion, as this treatment inactivates the restriction enzymes.

Aliquots (3 μl) of both digested and undigested DNA were then loaded on an agarose gel to test the successful effectiveness of the restriction digestion; the loading mixture contained the components shown in **Table 2.4**.

**Table 2.4.**

Reagent	Undigested vector	Digested vector
Vector	3 μl	3 μl
6 x loading dye	3.3 μl	3.3 μl
Milli-Q H <sub>2</sub> O	3.7 μl	3.7 μl

The contents of each tube were vortexed and loaded on a 1% agarose gel (1 x TAE).

#### **2.1.3.2. Preparation of an Agarose Gel in 1 x TAE Buffer**

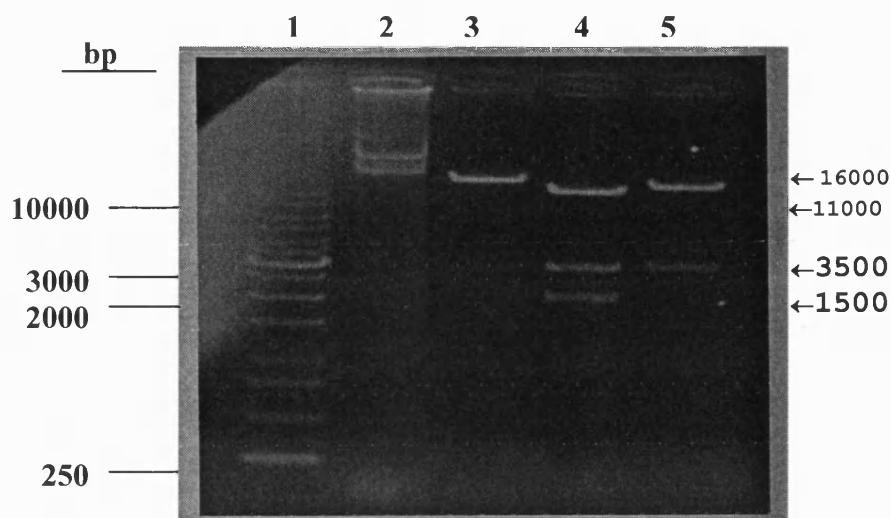
100 ml of 1% agarose gel was prepared as follows:

One gram of electrophoresis grade agarose gel (Roche, Switzerland) was dissolved in 100 ml final volume of 1 x TAE buffer to produce a 1% mixture, using a microwave oven for 2-3 min. The solution was cooled to about 60°C and poured into a casting tray. A comb was placed in the tray perpendicularly to allow the production of wells for loading of DNA samples. The width of each tooth of the comb was 2 mm and the spaces between the teeth were 0.1 mm. The gel was allowed to set at room temperature for 30 min. The gel was given a further 10 min in the cold room (4°C) to solidify. The comb was removed carefully to reveal the wells.

#### **2.1.3.3. Agarose Gel Electrophoresis**

The gel tray was mounted in an electrophoresis tank (Bio-Rad mini-sub DNA cell) and submersed in 1 x TAE buffer.

3 µl of marker, 10 µl of undigested mixture and 10 µl of digested mixture were then loaded into separate wells in the agarose gel. Electrophoresis was carried out at 120 V for 1h to obtain full separation of the bands. For the staining process the electrophoresis gel was removed and stained in a tank containing 1% ethidium bromide in 1 x TAE buffer for 10-15 minutes. The gel was visualized under UV light and photographed using a Polaroid MP4 camera and Polaroid 637 film. This gel is shown in **Figure 2.2**.



**Figure 2.2. Test of digestion of p706/HO4.7 with *KpnI*-*BglII* on a 1% agarose gel (1 x TAE).** Following digestion of p706/HO4.7 kb with the restriction enzymes *KpnI* and *BglII* alone or combined, the DNA was separated on a 1% agarose gel (1 x TAE). Lane 1 contains the 1 kb ladder. Lane 2 contains the undigested p706/HO4.7 kb plasmid. Lane 3 contains the product of digestion of p706 with *KpnI* restriction enzyme. Lane 4 contains the products of digestion of p706/HO4.7 plasmid with *BglII* and *KpnI* restriction enzymes and lane 5 contains the products of the digestion of p706/HO4.7kb with *BglII* enzyme.

#### **2.1.3.4. Ethanol Precipitation of the Products of the Enzymatic Digestion**

To 97  $\mu$ l of the reaction mixtures were added 10  $\mu$ l of 3 M sodium acetate (pH 7.5) and 200  $\mu$ l of absolute ethanol. The contents of the tubes were then left overnight at -20°C or for 15 min in an ethanol-dry ice bath. The DNA solutions were then centrifuged at 14000 rpm (10000 x g) for 30 min at 4°C. The supernatants were

discarded and 50  $\mu$ l of 70% ethanol was added to each tube to wash the DNA pellets. Following a further centrifugation at 14000 rpm (10000 x g) for 10 min (4°C), the supernatants were discarded and the pellets were air-dried for 5 min. The DNA pellets were resuspended in 45  $\mu$ l TE buffer.

#### **2.1.3.5. Digestion of the p706/HO4.7/*KpnI* Fragment with the *BglII* Restriction Endonuclease**

The digestion reaction contained the components shown in **Table 2.5**.

**Table 2.5.**

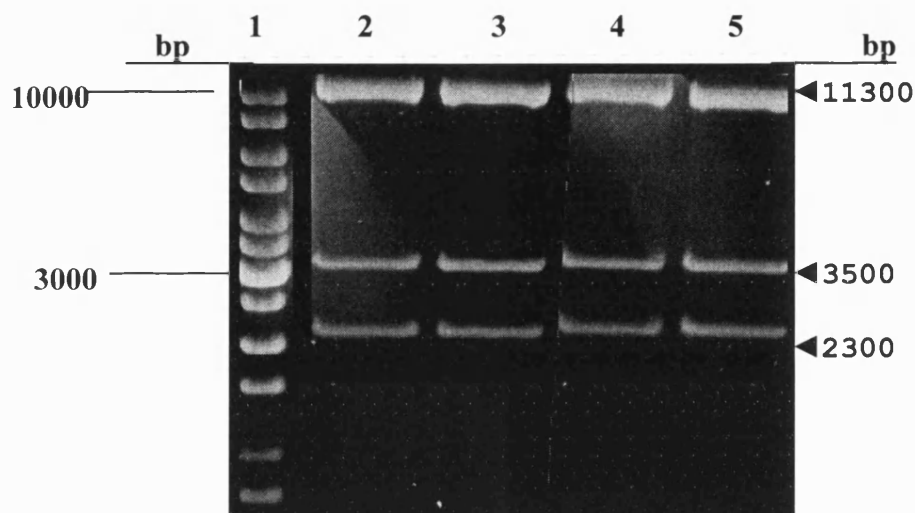
Reagent	Volume ( $\mu$ l)
DNA (p706/HO4.7/ <i>KpnI</i> )	45 $\mu$ l (= 20 $\mu$ g)
<i>BglII</i> enzyme	80 units
10 x REACT® 3 buffer	10 $\mu$ l
Milli-Q H <sub>2</sub> O	37 $\mu$ l
Total	100 $\mu$ l

The contents of each tube were mixed and incubated in a water bath at 37°C overnight.

#### **2.1.3.6. Electrophoresis of the Digestion Mixture**

A 1% agarose gel was prepared as described above and then placed into the electrophoresis tank containing 1800 ml of 1 x TAE and 10  $\mu$ l of 1% ethidium

bromide. The electrophoresis was carried out at 20 V overnight (16-18 h) at 4°C to separate the DNA bands. The gel was visualized under UV light and photographed using a Polaroid MP4 camera and Polaroid 637 film as shown in **Figure 2.3**.



**Figure 2.3. Test of digestion of p706/HO4.7 with *KpnI*-*BglII* on a 1% agarose gel (1 x TAE).** Following digestion of p706/HO4.7 kb with the restriction enzymes *KpnI*, and *BglII*, the DNA was separated on a 1% agarose gel (1 x TAE). Lane 1 contains the 1 kb ladder and lanes 2, 3, 4 and 5 contains the products of digestion of p706/HO4.7 plasmid with *BglII* and *KpnI* restriction enzymes performed in 4 replicates.

#### **2.1.3.7. Extraction and Purification of the p706/*KpnI*-*BglII* Fragment from the Gel**

The DNA bands (i.e. p706/*KpnI*-*BglII*, 11.3 kb) were cut from the gel using a scalpel blade and extracted using the QIA X-extraction kit (QIAGEN, UK), in accordance with the manufacturer's instructions.

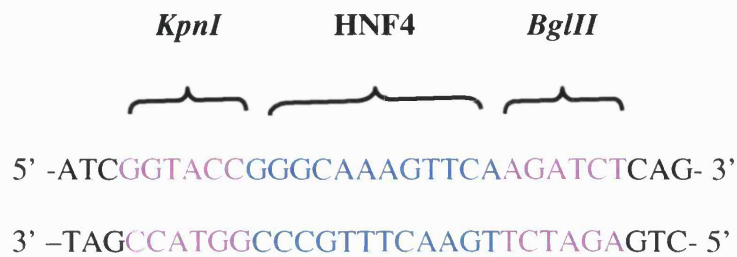
#### **2.1.4. Preparation of the “insert” DNA**

The purpose of this section was to design and prepare a series of 1, 3 and 5 repeat of HNF-4 responsive elements to be inserted within the *BglII-KpnI* sites of the p706/*KpnI-BglII* fragment vector. Subsections 2.1.4.1. to 2.1.4.4 describe in further detail the preparation of the HNF-4 responsive elements as “inserts”.

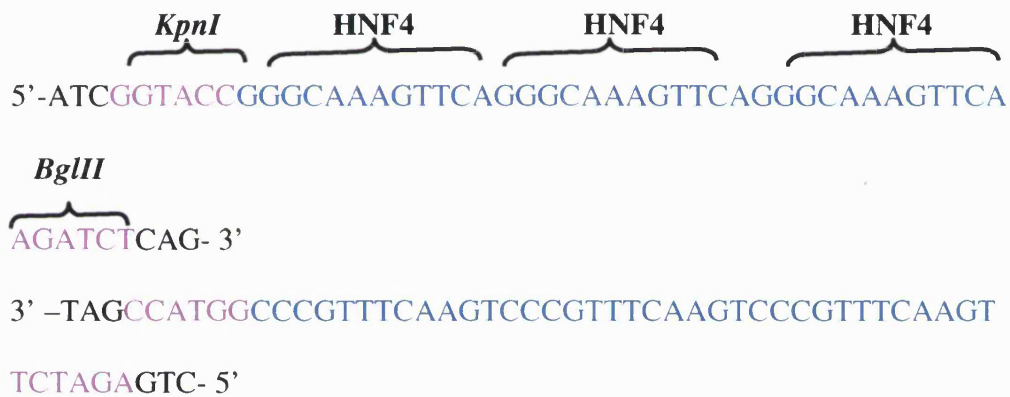
##### **2.1.4.1. Design of the Oligonucleotides**

The complementary oligonucleotides coding for the HNF-4 responsive element repeats were designed in such a way that when they annealed to each other they would produce the restriction sites *KpnI* at the 5'-end and *BglII* at the 3'-end. To further protect the restriction sites, 3 random additional bases were added at the two extremities of the designed oligonucleotides. These oligonucleotides were then purchased from (Sigma ,UK) as HNF4-1', HNF4-3', HNF4-5' with the following sequences:

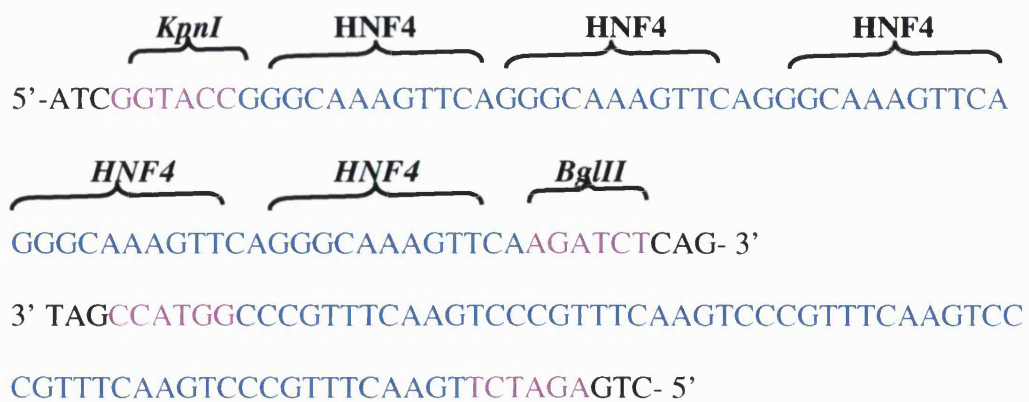
**HNF4-1`:**



**HNF4-3`:**



**HNF4-5`:**





#### **2.1.4.2. Annealing of the Oligonucleotides**

To anneal the complementary strands, 1 µg of each oligonucleotide was placed into a microcentrifuge tube and to this, 2 µl of Tris-HCl buffer (pH 7.5), 20 µl of 100 mM EDTA (pH 8.0) and 40 µl of 0.5 M NaCl were added. The volume was then made up to 200 µl using autoclaved Milli-Q water. The reaction was incubated at 96°C for 3 min and allowed to cool gradually to room temperature. The mixture was then stored at -20°C until subjected to polyacrylamide gel electrophoresis.

#### **2.1.4.3. Purification and Isolation of the Annealed Oligonucleotides**

To separate the annealed oligonucleotides from un-annealed or partially annealed material, the mixture was subjected to polyacrylamide gel electrophoresis

##### *2.1.4.3.1. Preparation of Polyacrylamide Gel Glass Plates*

Two 18 cm<sup>2</sup> glass plates were placed one on top of the other separated by 1 mm thin plastic spacers on three sides. The edges of the three sides were sealed using 3% molten agarose gel and the edges were fixed using three clips in each side.

##### *2.1.4.3.2. Preparation of the Non-denaturing Polyacrylamide Gel*

A 15% polyacrylamide (AccuGel, Flowgen, UK) gel was prepared as follows: 22.5 ml of 40% AccuGel (40%, 29:1, acrylamide: Bis-acrylamide) was placed in a beaker; to this 6 ml of 10 x TBE (see section A.1.8) and 31.5 ml autoclaved Milli-Q H<sub>2</sub>O were added. The mixture was placed for 3 min in a water bath at 37°C and then cooled in an icebox for 10 seconds.

#### *2.1.4.3.3. Casting of the Polyacrylamide Gel*

To the 60 ml of the 15% acrylamide solution were added 250 µl of 10% ammonium persulfate (APS) and 48 µl TEMED. Mix the contents.

The solution was poured between the two glass plates. A comb with 8 mm wide teeth and 0.2 mm spaces between the teeth was placed perpendicular with teeth down between the two glass plates and allowed to polymerize for 1 h at room temperature and for 10 min at 4°C. The comb was then removed to reveal the wells.

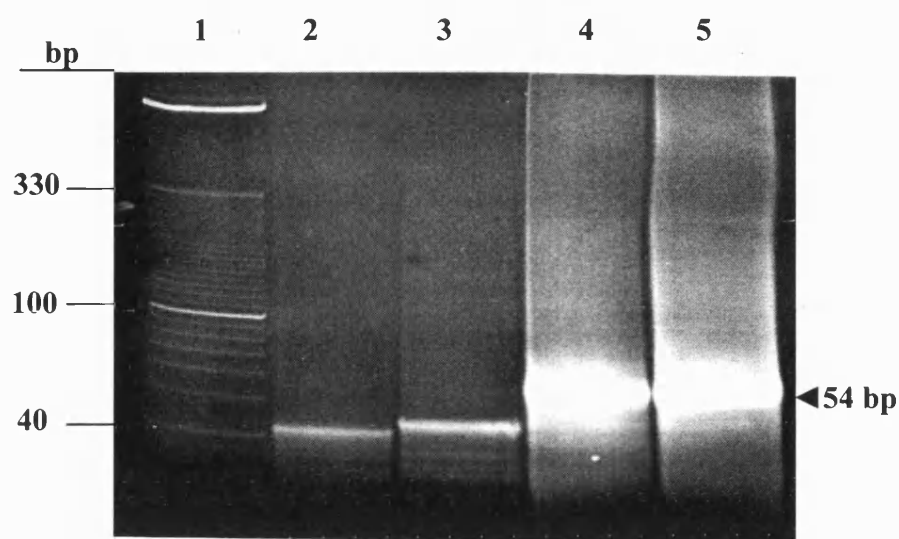
#### *2.1.4.3.4. Polyacrylamide Gel Electrophoresis*

Eight hundred ml of 1 x TBE buffer was poured into a vertical electrophoresis tank.

The gel was immersed in the TBE buffer at the bottom of the tank and fixed with two clips in either side. The wells in the upper part of the polyacrylamide gel were filled and covered with TBE buffer. Prior to loading the samples, the gel was pre-run for 1 h at 250 V.

Twenty µl of 6 x orange/blue loading dye (Promega, UK) were added to the annealed insert DNA and mixed. The order of loading of the samples to the gel was as follows: 100 µl of the annealed mixture, 10 µl (0.5 mg/ml) of 10 bp DNA marker, 3 µl (3700 ng) of sense insert DNA mixed with 7 µl of 6 x orange/blue loading dye, 3 µl (3700 ng) of anti-sense insert DNA mixed with 7 µl of 6 x orange/blue loading dye.

The polyacrylamide gel was then run at 250 V for 4 h at room temperature to separate the bands. The gel was stained by immersing the gel for 20-30 min in a 0.5 µg/ml ethidium bromide solution as described before. The gel was then removed and placed under UV light box to visualize the DNA bands. Photographs were taken using a Polaroid MP4 camera and a Polaroid 637 film as show in **Figure 2.4**.

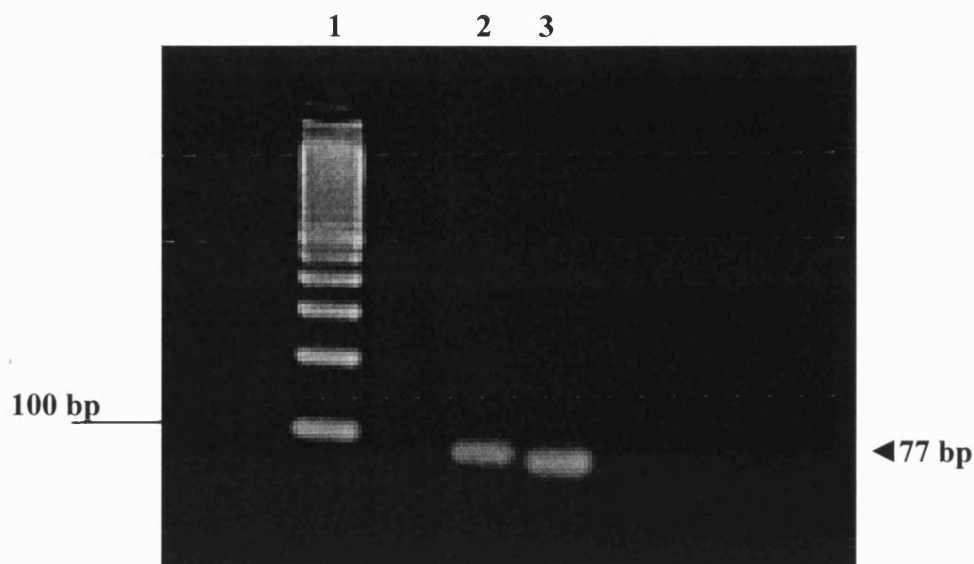


**Figure 2.4. Polyacrylamide gel electrophoresis of annealed oligonucleotides.**

Samples were loaded onto a 15% acrylamide gel and run for 4 h at 250 V. Lane 1 contains a 10 bp ladder. Lanes 2 & 3 contains free complementary strands of HNF4-3' and lanes 4 & 5 contain the annealed oligonucleotides of HNF4-3' to form double strand fragments.

The annealed DNA bands were excised from the gel using a scalpel and transferred into 1.5 ml microcentrifuge tubes. These bands were then purified from the gel using a QIAGEN extraction Kit (QIAGEN, UK), in accordance with the manufacturer's instructions. Finally, annealed DNA samples were resuspended in 30  $\mu$ l of TE buffer.

Two microlitres of the recovered double strand DNA inserts were then subjected to electrophoresis using a 3% agarose gel, to monitor the presence of annealed DNA fragments, and photographs were taken as shown in **Figure 2.5**.



**Figure 2.5. Analysis of the annealed DNA fragments in a 3% Agarose gel.** Lane 1 contains a 100 bp DNA ladder. Lanes 2 & 3 contain the annealed DNA samples HNF4-5'.

#### **2.1.4.4. Restriction of the Annealed DNA Fragments**

To prepare the annealed DNA fragments for ligation into the p706/*KpnI*-*BglII* vector, it was necessary to digest them with the restriction enzymes *KpnI* and *BglII*. This was achieved with two consecutive sets of digestions as follows:

##### *2.1.4.4.1. Restriction of the DNA “inserts” with Kpn I Restriction Endonuclease*

The first step was to digest the annealed DNA fragments with *Kpn I* restriction enzyme as detailed in **Table 2.6**.

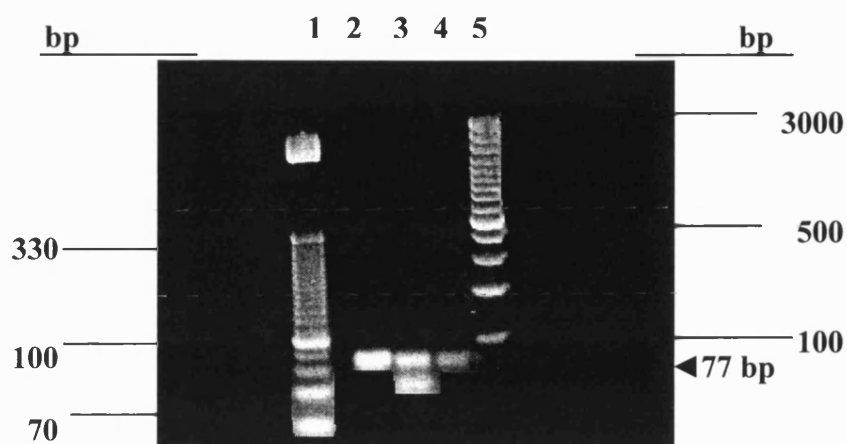
**Table 2.6.**

Reagent	Volume (μl)
“Insert” DNA	20 μl (=700 ng)
<i>KpnI</i> enzyme	5 units
10 x REACT® 4 buffer	3 μl
Milli-Q H <sub>2</sub> O	6.5 μl
Total volume	30 μl

The solution was incubated at 37°C overnight as described before.

*2.1.4.4.2. Ethanol Precipitation of the “insert” DNA*

To increase the yield of precipitation of DNA by ethanol, the ethanol precipitation was performed in the presence of 2.5 μl of 1 M MgCl<sub>2</sub> and 1.5 μl of 2.5 μg/μl tRNA as a carrier. The pellets were resuspended in 30 μl of TE buffer. Samples were then loaded in a 3% of high resolution MetaPhor® agarose gel (FMC Bioproducts, UK) and run at 20 V in 0.5 X TBE (4°C) for 3 hours. Next, the gel was stained with ethidium bromide and photographed under UV light. An example of such analysis is shown in **Figure 2.6**.



**Figure 2.6. Test of digestion of the ‘insert’ DNA fragments with *KpnI* and *BglII* restriction enzymes.** Samples were loaded on a 3% Metaphore agarose gel and run for 3 h at 20 V. Lane 1 contains the 10 bp DNA ladder. Lane 2 contains the undigested annealed insertion DNA (HNF4-5'). Lane 3 contains the insertion DNA digested with *KpnI* which resulted in 2 fragments (i.e. the insert DNA digested with *KpnI* and the tRNA band that was used as a carrier during ethanol precipitation). Lane 4 contains the product of digestion of the insertion DNA with *KpnI* and *BglII* enzymes, and lane 5 contains the 100 bp DNA ladder.

#### 2.1.4.4.3. Restriction of the “insert” DNA Fragments with the *Bgl II* Restriction Endonuclease

Following the digestion of the ‘insert’ DNA fragments with *KpnI*, the inserts were subjected to a second set of digestion with *BglII* to generate a *BglII* restriction site.

The digestion reaction contained the components shown in **Table 2.7**.

**Table 2.7.**

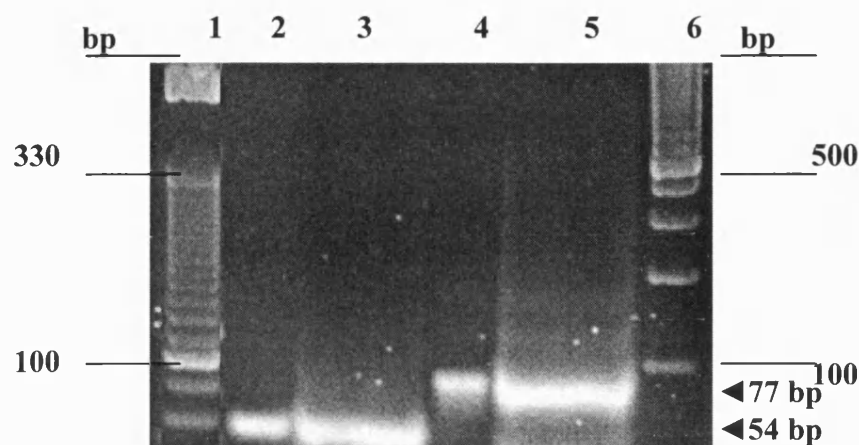
Reagent	Volume (μl)
Insertion DNA	25 μl (= 700 ng)
<i>Bgl</i> III enzyme	5 units
10 x REACT®3 buffer	3 μl
Milli-Q H <sub>2</sub> O	1.5 μl
Total volume	30 μl

The digestion was carried out at 37°C overnight as described before.

*2.1.4.4.4. Extraction and Purification of the “insert” DNA Fragments Following Digestion with Restriction Enzymes KpnI and BglIII.*

High-resolution 3% MetaPhor® agarose gels were produced by dissolving the MetaPhor® in 0.5 x TAE buffer at a low temperature in a microwave oven for 10 min. Electrophoresis was carried out at 20 V in the cold room at 4°C for 3 h to give full separation of the bands.

At the end of the run, gels were stained with ethidium bromide in 0.5 x TAE for 10 minutes. The DNA bands were visualized using a UV light box. Photographs were taken of the gel as shown in **Figure 2.7**.



**Figure 2.7. Analysis of the “insert” DNA fragments prior to extraction and purification.** Following digestions, the samples were loaded on a 3% Metaphor agarose gel for 3 h at 20 V. Lane 1 contains the 10 bp DNA ladder. Lane 2 contains the undigested annealed “insert” DNA (HNF4-3'). Lane 3 contains the “insert” DNA (HNF4-3') digested with *Kpn I* and *Bgl II*. Lane 4 contains the undigested annealed “insert” DNA (HNF4-5'). Lane 5 contains the “insert” DNA (HNF4-5') digested with *Kpn I* and *Bgl II* and lane 6 contains the 100 bp DNA ladder.

Bands from lane 3 and 5 were excised from the agarose gel and purified using the QIAGEN extraction Kit (QIAGEN, UK) protocol. The yield of the eluted material was then estimated by two methods, i.e. either by measuring the absorbance of DNA at 260 nm and 280 nm using the GeneQuant II spectrophotometer as described before or with ethidium bromide dot analysis.



### 2.1.5. Ligation

The concentration of the “insert” and the vector DNA to be used in the ligation were calculated. For every ligation two ratios of vector: insert DNA were tried, i.e.1:1 and 1:3. The latter ratio was found to be optimum. The following equation was used to calculate the quantity (i.e. ng) of the “insert” DNA required:

$$\text{ng of insert} = \frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of } \frac{\text{insert}}{\text{vector}}$$

The ligation reaction mixture is shown in **Table 2.8.**

**Table 2.8.**

Reagent	Volume (μl)
Vector DNA	105 ng
Insert DNA	105 (ratio1:1) or 300ng (ratio 1:3)
Ligase 10 x buffer	3 μl
T4 DNA ligase (Boehringer, 1unit/μl)	1 μl
Milli-Q H <sub>2</sub> O to	30 μl

A number of controls were included in every ligation:

- Vector, no insert DNA, no ligase enzyme, but plus ligase buffer and Milli-Q water.
- Vector, no insert DNA but plus ligase enzyme, ligase buffer and Milli-Q water.
- Vector, insert DNA (ratio 1:3), no ligase enzyme, but plus ligase buffer and Milli-Q water.
- Vector, insert DNA (ratio 1:1), no ligase enzyme but plus ligase buffer and Milli-Q water.

Where insert DNA or ligase enzyme was not included in the reaction mix the volume was made up to 10 µl with the addition of a greater proportion of Milli-Q water.

The reaction mixes were incubated overnight (16 h) at 12°C. Next morning 5 µl of each ligation mixture containing the ligated DNA (i.e. p706/HNF4-1', p706/HNF4-3' or p706/HNF4-5') was transformed into JM109 competent cells using the protocol outlined in section 2.1.6.

For transformation reactions, in addition to the ligation control, two additional controls were used. The first was a negative control and involved the transformation procedure without the addition of DNA (replaced by water) and the second, a positive control, where uncut vector was transformed.

#### **2.1.6. Preparation of Competent *E. coli* JM109 Cells**

*Escherichia coli* (*E. coli*) strain JM109 (Promega, UK), the host strain was used for all of the plasmids. Competent *E. coli* cells were prepared as follows:

A single colony of *E. coli* JM109 was picked from a fresh agar plate and inoculated into 5 ml of LB medium and incubated at 37°C overnight in a shaking incubator. The following morning, 1 ml of culture was diluted to 100 ml with LB and grown under standard conditions until the optical density (OD at 550 nm) of the solution reached 0.48 (2.5-3 h). The cell suspension was then chilled on ice for 5 min and centrifuged in sterile falcon tubes for 5 min, 6000 rpm (Beckman J2-MC) at 4°C. The supernatant was discarded and the cells re-suspended in 40 ml of Tfb I buffer (see section A.1.11) (2/5<sup>th</sup> volume of culture) and chilled on ice for 5 minutes. Cells were then centrifuged at 6000 rpm (Beckman J2-MC) for 5 min at 4°C and the supernatant discarded. Cells were subsequently re-suspended in 4 ml (2/25<sup>th</sup> volume of culture) of Tfb II buffer (see section A.1.12) and chilled on ice for 15 minutes. Cells were stored in 200 µl aliquots, snap frozen in a dry-ice/methanol bath and stored at -70°C until required.

#### **2.1.7. Transformation of Competent *E. coli* JM109 Cells with the Vectors p706/HNF4-1', p706/HNF4-3' and p706/HNF4-5'.**

Two hundred microlitres of competent cell suspension were thawed at room temperature and then placed on ice for 10 minutes. Plasmid DNA (<100 ng) was then added and gently mixed using a Gilson tip followed by incubation on ice for 30-45 minutes. The cells were heat shocked by incubation at 43°C for 45 sec and then returned to ice for 2 minutes. The cell suspension was then diluted to 800 µl with LB broth followed by incubation at 37°C for one and a half hours. After this, 100 µl of the cell culture was plated out onto a LB agar plate containing ampicillin at a concentration of 100 µg/ml. Plates were incubated overnight at 37°C.

### **2.1.8. Colony Picking and Small-Scale Plasmid Purification**

After checking the transformation controls, a sterile wire loop was used to transfer single colony forming units from the LB plates into 10 ml of LB broth containing the appropriate antibiotic. The cultures were grown overnight at 37°C on a shaking incubator at 300 rpm. The next day 10 ml of the LB broth containing the vector of interest was inoculated into 250 ml of LB broth containing the same antibiotic and the cultures were grown overnight at 37°C on a shaking incubator.

Plasmid DNA was purified using a modified version of the alkaline lysis method described by Sambrook *et al* (1989).

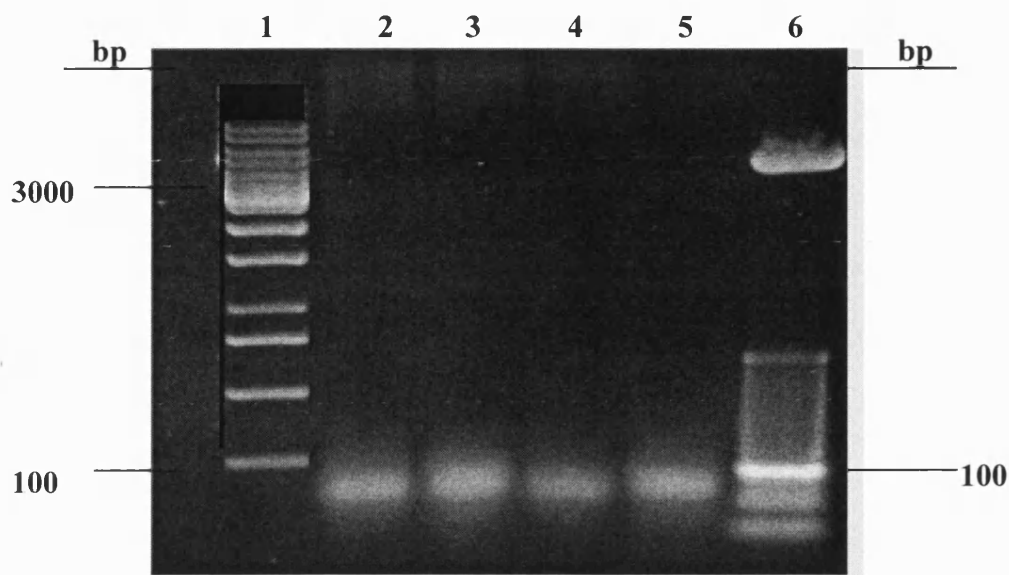
### **2.1.9. Polymerase Chain Reaction**

To check that the purified plasmids contained the HNF4-repeats as ‘inserts’, the DNA constructs were subjected to polymerase chain reaction (PCR) to amplify the region containing the HNF-4 inserts. For this purpose, two sets of oligonucleotides i.e., ‘sense’ (Right primer 5’ see section 2.1.4.1) and ‘anti-sense’ primers (Left primer 3’ see section 2.1.4.1) encompassing the region surrounding the insert were designed. Then, a master mix combining multiples of the reaction components listed below was assembled as outlined in **Table 2.9**. This master mix reduced variation when working with multiple DNA samples. The master mix was in a final volume of 50 µl. Care was taken to completely thaw and through mix the MgCl<sub>2</sub> stock.

**Table 2.9.**

	Volume per 50µl reaction	Final concentration
10 x PCR buffer	5 µl	1 x
MgCl <sub>2</sub> 50 mM	2 µl	2 mM
dNTPs 10 mM	1 µl	200 µM
Right primer 10 µM	1 µl	0.5 µM
Left primer 10 µM	1 µl	0.5 µM
Platinum <i>Taq</i> DNA polymerase (5 U/µl)	0.5 µl	0.05 U/µl
Plasmid DNA	1 µl	0.2 µg
Milli-Q H <sub>2</sub> O	50 µl	

The polymerase reaction consisted of the following stages, 94°C for 5 min (1 cycle); followed by three 1 min steps at 94°C, 50°C and 72°C (25 cycles); and finally 72°C for 7 min (1 cycle) using a Crocodile II temperature cycle (Perkin Elmer, UK). This protocol was based on the *Taq* DNA polymerase method (Chou *et al.*, 1992). The PCR sample was then run on a 3% agarose gel and photographed under UV light as shown in **Figure 2.8**.



**Figure 2.8. PCR analysis of the p706/HNF-4 constructs.** Following the PCR reaction, an aliquot of the PCR products were run on a 3% agarose gel for 1 h at 90 V. Lane 1 contains the 1 kb ladder. Lanes 2 to 5 contain the PCR products of p706/HNF4-5' plasmids and lane 6 contains the 25 bp ladder.

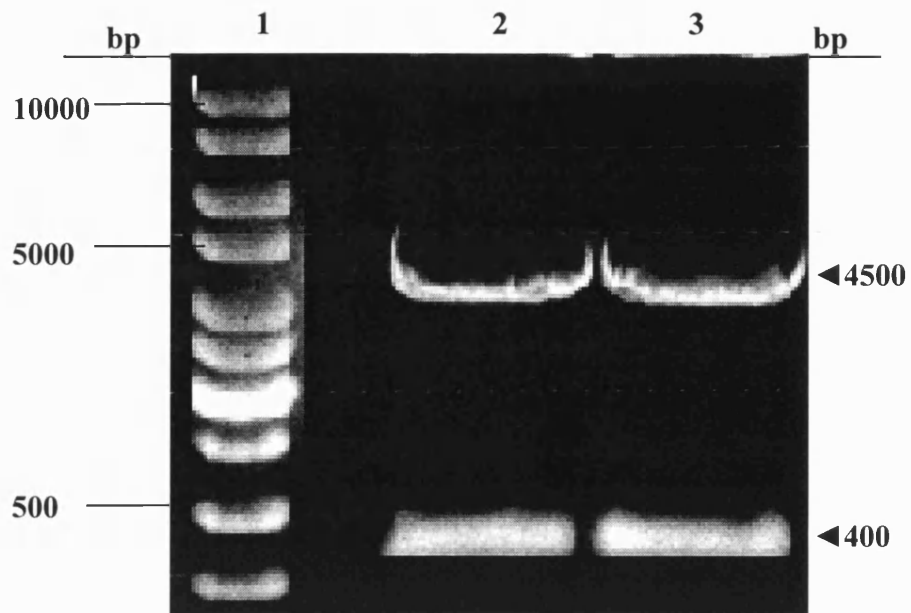
#### **2.1.10. Sequencing**

To further verify that the p706/HNF-4 constructs contain the expected HNF-4 sequence and repeats, DNA sequencing was carried out at the automated DNA sequencing facility in the Department of Biology and Biochemistry at the University of Bath using the Wisconsin Genetics Computer Group (GCG) software package on the University of Bath GENOME Unix server. The DNA samples for sequencing were supplied in 200 µl PCR tubes and contained 200-500 ng of DNA (plasmid), 10

pmol of the specific primers designed for the vector. The total volume of the samples was made up to a volume of 6 µl with Milli-Q water.

#### **2.1.11. Insertion of the SV40 Promoter into p706-Derived Plasmids**

Since the p706/HNF-4 plasmids lacked eukaryotic functional promoter elements for successful expression of the luciferase gene, it was necessary to incorporate a Simian virus 40 (SV40) promoter that contained the RNA polymerase II elements for initiation of transcription, upstream of the “*luc+*” and downstream of the HNF-4-enhancer elements. For this purpose, the p706/HNF4-1', 3' or 5' constructs were digested with *BglIII* and *HindIII* restriction enzymes as described before. Meanwhile, the SV40 promoter from pGL3 promoter (Promega, UK) vector was restricted and gel purified as shown in **Figure 2.9**. This was followed by ligation of the SV40/*BglIII-HindIII* promoter fragment into the p706/*BglIII-HindIII* fragment. The resulting plasmid constructs were called as p706/SV40 i.e. p706 lacking the 4.7 kb HO promoter but containing the SV40 promoter, p706/HNF4-1'/SV40, p706/HNF4-3'/SV40 and p706/HNF4-5'/SV40 i.e. p706/HNF4 plasmids all containing the SV40 promoter.



**Figure 2.9. Test of digestion of pGL3 with *KpnI-HindIII* on a 1% agarose gel.** Following digestion, the DNA samples were separated on a 1% agarose gel (1 x TAE) for 16 h at 20 V. Lane 1 contains the 1 kb DNA ladder. Lane 2 & 3 contain the products of digestion of pGL3 promoter plasmid with *KpnI* and *HindIII* in duplicate.



## ***B. Cell Culture.***

All biochemicals were of analytical grade and purchased from Sigma Chemicals, UK, except where indicated.

All the solutions used for the preparation of culture medium for mammalian cells were obtained from Gibco BRL, (Life Technologies, UK) except where indicated.

### **B.1. Cell Culture Solutions**

#### **B.1.1. Water**

Fresh Milli-Q water was used for the preparation of all culture media and solutions and was obtained from a Milli-Q PF Plus system with ultrafiltration cartridge (Millipore UK Ltd.).

#### **B.1.2. Phosphate Buffered Saline**

Phosphate buffered saline solution (PBS) tablets without magnesium or calcium ions were obtained from (Oxoid Ltd, UK). One tablet was dissolved in 100ml of Milli-Q water with a final pH of 7.3. Solutions were sterilised by autoclaving at 121°C for 15 minutes.

#### **B.1.3. Trypsin-EDTA**

0.05% (w/v) of Trypsin and 0.02% (w/v) EDTA.

#### **B.1.4. Culture Media Additives**

The culture media for human cells used in this study was the Dulbecco's Modified Eagle's Medium (DMEM) that was purchased as 1 x concentration. They were supplied with glutamax-1, sodium pyrovate, 1000 M G/L glucose, pyrodoxin and contained phenol red as an indicator.

#### **B.1.5. Animal Sera**

Foetal Bovine Serum (FBS), heat inactivated.

#### **B.1.6. Antibiotics and Antimycotics**

##### **B.1.6.1. Penicillin/ Streptomycin**

These antibiotics were dispensed as 5 ml vials at concentrations of 10 units/ml penicillin (penicillin G, sodium salt) and 10 µg/ml streptomycin (streptomycin sulphate) per milliliter of normal saline.

##### **B.1.6.2. Fungizone**

The fungizone is an antimycotic reagent comprised of a solution of 0.5 µg/ml amphotericine B, prepared in Milli-Q water.

##### **B.1.6.3. Hygromycin B**

This product was purchased at a concentration of 50 mg/ml in PBS.

Hygromycin B is an aminoglycosidic antibiotic produced by streptomyces hygroscopicus that kills bacteria and fungi.

### **B.1.7. Miscellaneous Products for Cell Culture**

#### **B.1.7.1. MEM Non-essential Amino Acids (MEM-NEAA)**

Non-essential amino acids were obtained as 100 x concentration, and they were supplied without L-glutamine.

#### **B.1.7.2. Opti-MEM®**

Opti-MEM®, is a serum-free growth medium specifically designed for transfection experiments.

#### **B.1.7.3. Trypan Blue**

Trypan blue, used for assessing cell viability, was obtained from BDH Laboratory Reagents Ltd. and was prepared as a 0.4% (w/v) solution in PBS.

#### **B.1.7.4. LIPOFECTIN® Reagent**

LIPOFECTIN® reagent was obtained as a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-[2,3-dioleyloxy] propyl]—N,N,N-trimethyl-ammonium chloride (DOTMA) in membrane-filtered water which is suitable for the transfection of DNA into tissue culture cells.

Growth media were prepared aseptically by adding to the 500 ml of DMEM (10% [v/v] FBS) 60 ml of FBS, 5 ml of Penicillin/ Streptomycin, 5 ml of MEM-NEAA, and 1 ml of Fungizone.

## **B.2. Cell Culture Equipment**

All aseptic techniques were carried out in a laminar flow cabinet (MDH Ltd) designed for vertical re-circulation of air. Cells were cultured in a LEEC PF2 humidified atmosphere at 37°C with 5% CO<sub>2</sub> (v/v) 95% air incubator (Laboratory and Engineering Company). Cells were visually assessed daily for evidence of microbial contamination under an inverted light microscope (WILD M4 Wild Heerbrugg Ltd).

## **B.3. Cell Culture Disposable Items**

Sterile 75 cm<sup>2</sup> and 175 cm<sup>2</sup> top tissue culture flasks were obtained from Fermentas, UK. Six and ninety-six well plates were obtained from Nunc, (Denmark). Cryopreservation ampoules used for storing frozen cells in liquid nitrogen were obtained from Corning, UK.

## **2.2. Cell Culture Methods**

### **2.2.1. Cell Lines**

**HepG2 cell line:** A human hepatocellular carcinoma cell line.

In the present study, this cell line was used as a model for liver cells. HepG2 cells were obtained from ATCC (USA), and were grown in DMEM medium containing 10% FBS, Penicillin/ Streptomycin and MEM non-essential amino acids. For routine culture, cells were seeded at a density of  $3 \times 10^5$  cells in 10 cm plastic plates and were grown either for 4 days to reach 80% confluency or for 5 days to reach

90% confluency. The medium was changed every 48 hours. The cells were passaged by trypsinisation once a week and were used for experiments.

**HtTA cells:** a human cervix carcinoma, Hela derivative and transformed cell line.

The HtTA cell line was derived from Hela cells being stably transfected with a tetracycline-controlled transactivator (tTA) consisting of the tet repressor fused with the activating domain of virion protein 16 of the herpes simplex virus (HSV).

HtTA was kindly obtained from the laboratory Professor Rex M. Tyrrell (Pharmacy and Pharmacology Department) and were grown in DMEM medium containing 10% FBS, Penicillin/ Streptomycin and MEM non-essential amino acids. Cells were seeded at a density of  $2 \times 10^3$  in 10 cm plastic plates and were grown for 2 days to reach 90% confluency. The cells were trypsinised for passage every 3 days and were used for experiments.

### **2.2.2. Subculturing of Cell Lines Using the Trypsinisation Technique**

For subculture, 175 cm<sup>2</sup> flasks containing the monolayers of each cell line were rinsed three times with 10 ml aliquots of PBS and then incubated at 37°C with 2 ml of 0.25% Trypsin solution for approximately 5 min. Detached cells were diluted to 20 ml with complete culture medium and were then centrifuged at 1000 rpm (700 x g) for 10 min at room temperature. The supernatants were discarded and the cell pellets were resuspended in 1ml of culture medium for a viable count using a hemocytometer. Next, an appropriate aliquot of each cell suspension was transferred to a sterile 175 cm<sup>2</sup> flask containing 50 ml of fresh culture medium.

### 2.2.3. Estimation of the Cell Numbers

To determine the number of cells, a viable count was performed using a hemocytometer. Following detachment of cells from the flask, the cell suspension was briefly vortexed to break up the clumps of cells. 12 µl of well-suspended cells were mixed with an equal volume of trypan blue solution. The sample was loaded into an etched glass chamber with raised sides that will hold a quartz coverslip 0.1mm above the chamber floor. Viable cells were detected by a bright 'halo' light around their cell membrane whereas dead cells were permeabilised by the dye and stained blue. The viable cell numbers in the four squares surrounding the central square were counted and this was repeated for the other side of the hemocytometer chamber, using an inverted light microscope. The viable cell concentration in the original suspension was calculated using the following equation:

$$\text{Cell concentration per milliliter} = \frac{\text{Total cell count in 4 squares} \times 5 \times 10^5}{\text{Number of squares (4)}}$$

### 2.2.4. Cell Storage and Recovery

Cells were prepared for storage by detachment of a confluent monolayer by trypsinisation as described above followed by centrifugation at 1000 rpm (700 x g) for 10 min. The supernatant was then discarded and the cell pellets were resuspended in the growth media at a concentration of  $2 \times 10^5$  cells/ml. A cell freezing solution was prepared in a filter-sterilised solution of 25 ml FBS supplemented with 10% (v/v) dimethylsulphoxide (DMSO) (spectrophotometric

grade) as a cryopreservative in a glass bottle. Next, 0.4 ml of cell suspension was dispensed in 1.8 ml aliquots with 0.6 ml of the freezing solution into Corning tubes, transferred to isopropylalcohol in  $-70^{\circ}\text{C}$  overnight, then fitted into a Union Carbide LR-40 liquid nitrogen freezer where they were stored at approximately  $-148^{\circ}\text{C}$ .

For cell recovery, the contents of an ampoule were thawed rapidly by brief incubation in a  $37^{\circ}\text{C}$  water bath followed by dilution with 10 ml of fresh medium. Cells were centrifuged for 10 minutes at 1000 rpm ( $700 \times g$ ) and the supernatant was discarded. The cells were then resuspended in 10 ml of fresh medium and then transferred into a  $175\text{cm}^2$  flask containing 50 ml of medium and then incubated under standard culture conditions. Cells were cultured for at least two passages in order to establish them before any experiments were carried out.

## **2.2.5. Transfection of DNA into the Cells**

### **2.2.5.1. Cell culture in 6-well Plates**

Following the determination of the concentration of the cell lines, the cells were transferred into 6-well plates. Cells were seeded at a density such that at the time of transfection monolayers were 40-50% confluent.

### **2.2.5.2. Preparation of Transfection Complexes**

#### *2.2.5.2.1. Preparation of Solution A*

For each transfection, a mixture of 2  $\mu\text{g}$  of DNA plasmid of interest (i.e. P706 derivatives) and/or 0.25  $\mu\text{g}$  of a reference plasmid (i.e. the  $\beta$ -galactosidase expression vector 'pCMV $\beta$ Gal' obtained from CLONTECH, UK) were diluted to 100  $\mu\text{l}$  with serum free medium Opti-MEM in a sterile 1.5 ml microcentrifuge tube, gently mixed and incubated for 30 min at room temperature. This cotransfection

was performed simultaneously to enable differences in transfection and growth to be corrected.

#### *2.2.5.2.2. Preparation of Solution B*

For each transfection, variable quantities of lipofectin reagent (i.e. 5-20  $\mu$ l) were diluted to 100  $\mu$ l with Opti-MEM, gently mixed and incubated for 30 min at room temperature.

Complexes were prepared by adding 100  $\mu$ l of solution B to 100  $\mu$ l of solution A. The complex solution was gently mixed by pipetting the solution up and down (i.e. 4 times). The mixture was then incubated at room temperature for 20 min. Meanwhile, monolayers of cells were washed once with warm Opti-MEM and 800 $\mu$ l of this medium were added to each well. 200  $\mu$ l of the complex solution were added to each well.

The transfection was then carried out for 4-5 hours at 37°C after which, the transfection medium was replaced with the fresh complete culture medium and cells were incubated for a further hour before harvesting for analysis.

For all transfection studies, each data point represents the mean  $\pm$  standard deviation ( $\pm$  SD) of triplicate samples and each experiment was repeated on at least three to four occasions.

#### **2.2.5.3. Addition of Hygromycin B**

To maintain the expression of hygromycin B resistant p706 and p706-derivative plasmids, following transfection hygromycin B was added to each well plate at a concentration of 150  $\mu$ g per ml of media.



## **2.2.6. Preparation of Cell Lysates for Further Assays**

### **2.2.6.1. Detergent Lysis Method**

After the appropriate period of post-transfection incubation, the cell monolayers were first washed twice with 2 ml of PBS and then treated with 115 µl of lysis buffer (see section A.1.10) per well followed by freezing at  $-70^{\circ}\text{C}$ . The next day, the samples were thawed at room temperature for 30 min and the cell extracts were scraped from the culture dish, collected and transferred into sterile 1.5 ml microcentrifuge tubes and centrifuged for 10 min at 1000 rpm (700 x g) and the supernatant was used for analysis.

### **2.2.7. Luciferase Reporter Assay**

Luciferase levels within cells were quantified using the Promega luciferase assay system (Promega, UK) according to the manufacturers instructions (Technical Bulletin, Promega, UK). As little as  $10^{-20}$  moles of firefly luciferase can be measured using this kit. A light unit versus relative enzyme concentration was constructed using purified firefly luciferase.

A series of solutions was prepared by dilution of purified luciferase (Promega, UK) stock with 10 ml assay buffer (Promega, UK). After centrifugation of the cell lysate (from section 2.2.6), 20 µl of room temperature cell extract (supernatant) were mixed with 100 µl of luciferase assay reagent (luciferase assay substrate +10 ml of assay buffer) again at room temperature, using luciferase reagent as a reference. The disposable test tube (Promega, UK) containing the reaction was quickly vortexed and then immediately placed into the luminometer (TD-20/20) to measure the light intensity produced. The instrument settings were as follows: sensitivity 25.1, delay 5 seconds, integration 10 seconds and repeats 3 times.

### 2.2.8. Protein Assay

The protein content in cell extracts was measured using the Bradford Assay (BioRad, Germany). Samples and standards were prepared according to the manufacture's protocol and calibration curves for protein were constructed using bovine serum albumin (BSA) as a standard. Briefly, the BSA stock solution (100 mg/ml, Roche) was first diluted in water to the final concentration of 1 mg/ml and then standards were prepared in tubes as indicated in **Table 2.10**.

**Table 2.10.** The standard curve using BSA as protein

Amount of BSA 1 mg/ml (µl)
0
2
5
7
10
12
15
17
20

The samples were prepared by mixing 20 µl of cell extract (supernatant) with 780 µl of H<sub>2</sub>O. Next, 200 µl of Bradford reagent was added to each tube and the mixture was immediately vortexed to reveal the formation of the blue colored Biorad/protein complexes. 100 µl of each tube was then added to a 96 well microplate and the

concentration of the proteins in the cell lysates was determined at 595 nm using the Dynatech MR5000 microplate-reader by the Biolinx-Rad, 2.1 software program. The protein concentrations were calculated by linear regression from the standard curve.

#### **2.2.9. $\beta$ -galactosidase Assay**

$\beta$ -galactosidase activity was determined 48 h following the cotransfection as follows: 50  $\mu$ l of cell extract was incubated with 150  $\mu$ l of reagent containing 2.5 mM Chlorophenol Red  $\beta$ -galactopyranoside (CPRG) (Boehringer Mannheim, Mannheim, Germany) and 1.25 mM  $MgCl_2$ . The activity of  $\beta$ -galactosidase was calculated from the absorbance at 550 nm obtained in the final reaction using a 96-well microplate reader (Dynatech MR5000).

#### **2.2.10. Green Fluorescence Assay**

Green fluorescent protein (GFP) is a bioluminescent protein that originates from *Renilla reniformis*. The bioluminescence is expressed as bright green waves of light that run across the surface of the cells. The source of this green light is the fluorescence from a protein containing an unusual chromophoric group.

To determine the efficiency of transfection in the cell lines used in this study, the plasmid pEGFP/CMV was used. In this plasmid, the 'enhanced green fluorescent protein (EGFP)' has been cloned downstream of the CMV (cytomegalovirus) promoter in pEGFP (4.7 kb), the enhanced green fluorescent protein vector was obtained from CLONTECH, UK. This vector was transfected into both the HepG2 and HtTA and cell lines fluorescence activated cell sorter (FACS) analysis was used

to quantify both the level of transfection and expression of the GFP in both cell lines.

Briefly, monolayers of HepG2 and HtTA cell lines grown on 10 cm plates were transfected with EGFP vector using the lipofectin and then incubated with the appropriate compounds for 48 hours. The media was then removed and saved. The cells were washed twice with PBS and incubated for 5 min with 3ml of 0.25% w/v trypsin. After detachment, 3 ml of saved media was reapplied to the cells and the cells were transferred to a flow cytometer tube (Becton Dickinson, UK). The cells were spin into a pellet by centrifugation at 1000 rpm (700 x g) for 5 min in a Jouan CR412 centrifuge. The supernatant was removed and replaced with 5 ml of PBS. The resultant suspension was then analysed on a (FACS) Vantage SE (Becton Dickinson, UK) using emission wavelength fluorescence at 535 nm under specific excitation at 435 nm.

## CHAPTER 3      RESULT 1

One-way of targeting gene expression *in vivo* is to control transcription using a tissue specific regulatory system. Tissue specific promoters or enhancers are used in transgenic animals and could be utilised in medicines for gene therapy.

In the introduction section (1.1.5.1.) we have discussed the transcription factors (TFs) that are enriched in the liver. At present the usual methods for selection of a tissue specific promoter is to identify a gene which is expressed at high level in the target tissue and then to use the promoter for this gene to drive the expression of another therapeutic gene in the target tissue. Alternatively one could examine the TF-binding sites within a population of known promoters to identify TFs that are associated with a target tissue/organ specific expression.

In the present study we have used both approaches to identify the TF-responsive elements associated with the liver specific expression.

In this section we describe how the following computer analyses allowed us to identify specific promoter TF-binding sites that are abundant in the liver.

1. First a literature search was carried out on human liver promoters using the **Gene Bank Database**. This led us to the identification of up to 50 promoters isolated from the human liver promoter sequences. This analysis also contained information about their access number, sequence and a short explanation about the origin of the species.
2. Data obtained from **Gene Bank** was then catalogued using **VECTOR NTI** program and the data were analysed in order to identify the promoters in the DNA sequences and the TF- binding sites for each promoter. **VECTOR NTI** is a software package that can integrate sequence analysis software

solution for molecular biologists, since it visualizes, constructs and stores analysis of biological molecules. It is therefore a convenient housekeeping system for storage of data.

3. Further analysis of the promoters was carried out using the **TFSEARCH** algorithms at the following web site [http://www.rwcp.or.jp/lab/adappl/papia.html]. The **TFSEARCH** is a Japanese program that allows the identification of the potential binding elements of TFs as shown in **Figure 3.1**. Furthermore, **TFSEARCH** identifies consensus sequences for each transcription factor using consensus data, which is available in the **TFMATREX** database as shown in **Figure 3.2**.

### TFSEARCH Search Result

```

** TFSEARCH ver.1.3 ** (c)1995 Yutaka Akiyama (Kyoto Univ.)

This simple routine searches highly correlated sequence fragments
versus TFMATRIX transcription factor binding site profile database
by E.Wingender, R.Knueppel, P.Dietze, H.Karas (GBF-Braunschweig).

<Warning> Scoring scheme is so straightforward in this version.
           score = 100.0 * ('weighted sum' - min) / (max - min)
           The score does not properly reflect statistical significance!

Database: TRANSFAC MATRIX TABLE, Rel.3.3 06-01-1998
Query:    PGL3 (650 bases)
Taxonomy: ALL
Threshold: 85.0 point

TFMATRIX entries with High-scoring:

1 CCCGGGAGGT ACCGAGCTCT TACGCGTGCT AGCTCGAGAT CTAAGTAAGC entry      score
                        ----->
                        <-----
                        <-----
                        ----->
                        M00263 StuAp 89.2
                        M00263 StuAp 87.7
                        M00123 c-Myc/ 86.8
                        M00048 ADRI 86.2

51 TTGGCATTCC GGTACTGTTG GTAAATGGA AGACGCCAAA AACATAAAGA entry      score
                        --- M00028 HSF 100.0
                        <----- M00100 CdxA 96.2
                        --- M00029 HSF 96.0
                        -----> M00148 SRY 90.9
                        ----- M00148 SRY 90.0
                        <----- M00050 E2F 87.4
                        <----- M00101 CdxA 87.1
                        <----- M00029 HSF 86.3
                        <----- M00253 cap 85.3

```

**Figure 3.1:** An example of the data obtained for TF-binding sites in **TFSEARCH** program.

```

AC M00028
XX
ID ISHSF_01
XX
DT 18.10.94 (created); ewi.
DT 16.10.95 (updated); ewi.
XX
NA HSF
XX
DE heat shock factor (Drosophila)
XX
BF T00386; HSTF; Species: fruit fly, Drosophila melanogaster.
XX
P0      A      C      G      T
01      31      14      4      1      A
02       0       0      50      0      G
03      48       2       0       0      A
04      43       1       5       1      A
05      23       1      14      12      N
XX
RA 50 functional genomic HSEs
XX
CC not included are sequences with more than 2 mismatches at positions
CC 2 to 4; the matrix only describes the properties of the basic
CC 5-bp unit of which three have to be present to constitute a minimal HSE
XX
RN [1]
RA Fernandes M., Xiao H., Lis J. T.
RT Fine structure analyses of the Drosophila and Saccharomyces
RT heat shock factor-heat shock element interactions
RL Nucleic Acids Res. 22:167-173 (1994).
XX
//

```

**Figure 3.2:** An example of the data obtained from TFMATREX program.

4. In this example, consensus sequences containing more than 6 nucleotides were considered to be “long sequences” whereas those having less than 6 nucleotides were considered to be “short sequences”. The assumption was that “long sequences” would be more specific elements. **Table 3.1** summarizes data obtained from the **TFSEARCH** on number of TF-binding sites having long sequence and **Table 3.2** summarizes the TF-binding sites having short sequences. The data obtained from the **TFSEARCH** also provide information about the number of TF-binding sites and their prevalence within the promoters studied. Both tables show the percentage of the promoters, which have at least one potential site for each TF.

**Table 3.1:** TF-binding sites having long sequences more than 6bp.

<b>Name</b>	<b>Total number</b>	<b>% Percentage</b>
<b>IRF-1</b>	3	18.75%
<b>NF-<math>\kappa</math>B</b>	2	12.5%
<b>EGR</b>	3	18.75%
<b>SP-1</b>	4	25%
<b>GC-box</b>	4	25%
<b>SREBP</b>	9	56.25%
<b>HNF-4</b>	10	62.5%
<b>CREBP</b>	5	31.25%
<b>C/EBP</b>	10	62.5%
<b>MYBPH</b>	4	25%
<b>EVI-1</b>	3	18.75%
<b>Oct-1</b>	8	50%
<b>HSF</b>	6	37.5%
<b>C-MYC</b>	2	12.5%



**Table 3.2:** TF-binding sites having short sequences less than 6 bp.

<b>Name</b>	<b>Total number</b>	<b>% Percentage</b>
<b>NIT2</b>	15	94%
<b>CdxA</b>	16	100%
<b>Cap</b>	16	100%
<b>BCD</b>	10	63%
<b>CF1</b>	4	25%
<b>HSF</b>	4	25%
<b>AP-4</b>	13	81%
<b>C/EBP</b>	15	94%
<b>HFH-2</b>	4	25%
<b>HNF-3b</b>	10	63%
<b>HNF-1</b>	14	88%
<b>GATA-2</b>	2	13%
<b>SRY</b>	11	69%
<b>DFd</b>	14	88%

5. A literature search was carried out using **BIDS**, to deduce which promoters are active in the liver but not in other organs. Sixteen of the promoters were thought to be liver specific; 34 others were known to promote expression of their corresponding genes in other organs. An attempt was made to examine the liver specificity of each response element by calculating the percentage of the promoters, which had each response element. A comparison was

made between the TF-binding elements present in liver promoters having long sequences with those present having short sequences as shown in **Tables 3.1 and 3.2**. If a TF-binding element was present in a high percentage of liver promoter with long sequences, but not in short group, it was assumed that these elements were worthy of further investigation.

6. Another separate approach to identify liver-specific promoters was to use **BIDS** to identify promoters for the 16 genes which are more commonly expressed in the liver i.e. promoters for albumin, cytochrome P450 isoenzymes, plus enzymes from hepatic mitochondria inner membrane compartment. The latter enzymes play an important role in the metabolism of cholesterol and the related sterols. The metabolic steps are carried out by hepatic mitochondria cytochrome P450 (Mullick, 1995). A survey of data in the gene bank revealed 19 promoters of cytochrome p450 isoenzymes. A comparison of these promoters was made as before using **TFSEARCH** and the data was compared with the previous 16 liver specific promoters.
7. A book called *TRANSCRIPTION FACTORS ESSENTIAL DATA* (Locker, 1996) is an attempt to present the essential data for each TF such as how it functions in regulating transcription, relationship to other proteins and where it is expressed, and includes some tables about tissue specific regulators. The suggested liver specific TF-binding sites are ANF, AT-rich, C/EBP, HLF, and HNF-4 as shown in **Table 3.3**.

**Table 3.3:** Potential liver specific TFs, where K= G or T, Y= C or T, W= A or T, R= A or G, N= Not defined.

<b>Factor</b>	<b>Consensus Binding Site</b>	<b>Family</b>	<b>Element</b>	<b>Name</b>
<b>ANF</b>	CTGTGGGAACA	Not cloned	ANF site	Atrial natriuretic factor
<b>C/EBP</b>	TKNNGYAAKAT TGCGAAT	BZIP/PAR	C/EBP site	Enhancer binding protein
<b>HNF-4</b>	KGCWARGKYC AY	ZnF	HNF4 site	Hepatocyte nuclear factor
<b>ATBF1-A</b>	ATTAATAATTAC	Hom/ZnF	AT-rich site	Zinc-finger homeodomain protein
<b>HLF</b>	GTTACGTAAT	BZIP/PAR	HLF site	Hepatic leukaemia factor
<b>TEF</b>	GTTACCTAAT	BZIP/PAR	VBP	Transcription enhancer factor

And other liver specific transcription factors that are not yet defined are shown in

**Table 3.4.**

**Table 3.4.** Liver specific TF's that are not well defined

<b>Element</b>	<b>Binding Site</b>	<b>Family</b>
<b>Conexin 32 gene</b>	AAGCTCCGGTCCT GGGCTGCTTGT	Not cloned
<b>SLP gene promoter element.</b>	TTCCGGGC	Not cloned

The analysis carried out in this study suggested that the most likely liver specific TFs are those listed in **Table 3.5**.

**Table 3.5.** Suggested liver specific transcription factors.

<b>Factor</b>	<b>Binding site</b>	<b>Family</b>	<b>Element</b>	<b>Name</b>
<b>HNF-4</b>	KGCWARGKYC AY	ZnF	HNF4 site	Hepatocyte nuclear factor
<b>C/EBP</b>	TKNNGYAAKAT TGCGAAT	BZIP/PAR	C/EBP site	Enhancer binding protein
<b>HLF</b>	GTTACGTAAT	BZIP/PAR	HLF site	Hepatic leukaemia factor
<b>TEF</b>	GTTACCTAAT	BZIP/PAR	VBP	Transcription enhancer factor

### **3.1. Discussion:**

In general there are hundreds of known TFs but it is assumed that in total there may be thousands of other TFs yet to be isolated. Hepatocyte-specific gene expression is determined by TFs of several families, which were initially identified by their binding sites in the regulatory regions of genes that are expressed specifically in the liver. These factors belong to four different families, including homeodomain homolog “HNF-1” (Frain *et al.*, 1989), the basic leucine zipper protein “C/EBP”, the

forkhead homolog “HNF3” (Lai, 1991), and an orphan steroid hormone receptor “HNF4” (Sladek *et al.*, 1990). All of these TFs were found to be expressed not only in the liver but also in the kidney, intestine, pancreas and spleen (Zaret, 1994). Cell-specific transcription of these factors is an important element of their distribution in specific cell types and the determination of expression is established requires knowledge of the regulation of the genes for these factors during embryogenesis (Xanthopoulos *et al.*, 1991).

## CHAPTER 4    Result 2

The unique phenotype of hepatocytes arises from the expression of genes in a liver-specific fashion, which is controlled primarily at the level of mRNA synthesis. By analyzing DNA sequences implicated in liver-specific transcription, it has been possible to identify members of the nuclear proteins such as the liver-enriched *trans*-activating factors, HNF-1, HNF-3, HNF-4, HNF6, C/EBP and DBP, which are key elements in the liver specific transcriptional regulation of genes. Each of these factors binds to unique DNA sequences (*cis*-acting elements) in the promoter and enhancer regions of genes expressed in terminally differentiated hepatocytes such as albumin. The determination of the tissue distribution of these factors and analysis of their hierarchical relations has led to the hypothesis that the cooperation of liver-enriched transcription factors with the ubiquitous *trans*-activating factors is necessary, and possibly even sufficient for the maintenance of liver specific gene transcription (see section 1.1.5.1. and Result 1 Table 3.5.).

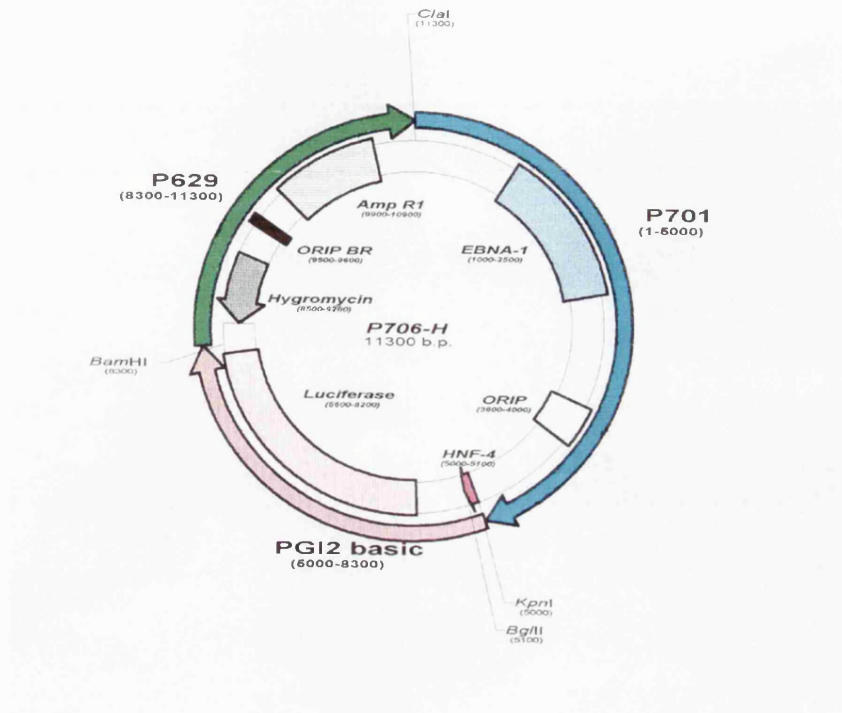
In the present study, the HNF-4 binding site was chosen as the most proven liver specific regulatory element (see Result 1) to test the hypothesis that a tandem repeat of these liver-enriched transcription factor binding sites linked upstream of a luciferase gene within a reporter plasmid would increase the basal level expression of the luciferase gene in a liver-specific cell line when compared to a non-liver cells.

To this regard we made several constructs using the EBV-based p706 plasmid that contained a series of 1', 3' and 5' repeats of HNF-4 binding sites upstream of the

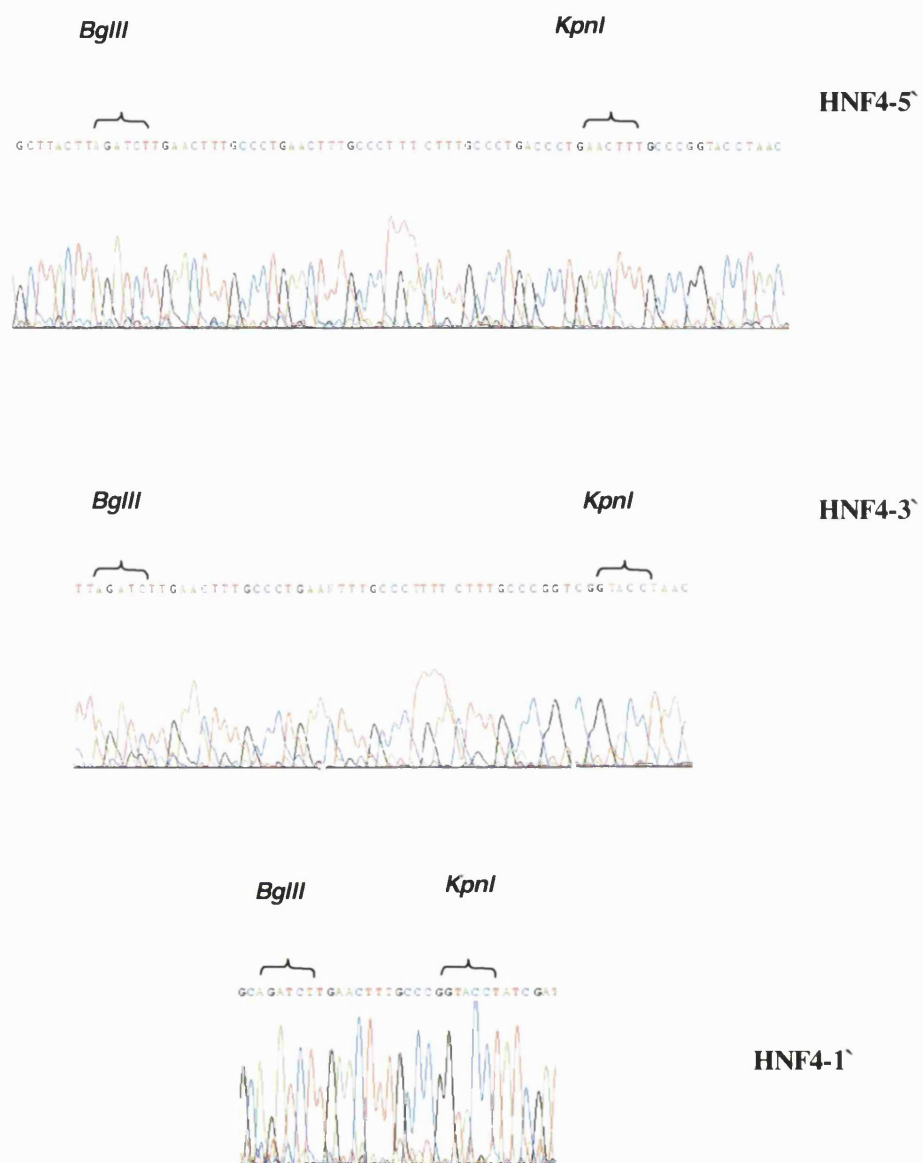
luciferase gene (i.e. after removing the 4.7 kb fragment containing the upstream promoter region of HO-1 gene, see 2.1.3). The resulting plasmids were called as p706/HNF4-1', p706/HNF4-3' and p706/HNF4-5', respectively. The restriction analysis confirmed that the 4.7 kb upstream promoter region of HO-1 was removed, reducing the size of p706 DNA plasmid from 16 kb to 11.3 kb (see 2.1.3). The sequencing confirmed that the ligation of HNF-4 into p706 was successful (see **Map 2 and Figure 4.1.**). Furthermore since the p706/HNF-4 plasmids lacked eukaryotic functional promoter elements for successful expression of the luciferase gene, it was necessary to incorporate a promoter (i.e. SV40 promoter from PGL3 promoter plasmid) upstream of the "*luc+*" and downstream of the HNF4 regulatory element(s). The resulting plasmid constructs were called as p706/SV40 (i.e. p706 lacking the 4.7 kb HO promoter but containing the SV40 promoter) as shown in **Map 3**, p706/HNF4-1'/SV40, p706/HNF4-3'/SV40 and p706/HNF4-5'/SV40 (i.e. P706/HNF4 plasmids all containing the SV40 promoter). The sequencing confirmed that the insertion of the SV40 into p706/HNF-4 plasmids was successful (see **Map 4 and Figure 4.2.**).

We then transiently transfected these vectors into two human carcinoma cell lines, HepG2 (liver-specific cells) and HtTA1 (Hela derived non-liver cells) and then monitored the level of luciferase expression within the lysates of transfected cells. Since EBV-based episomal vectors have the advantage of autonomous replication and their expression can be maintained for several days under hygromycin, the basal level expression of luciferase in these constructs was followed up to 5 days following transient transfection.

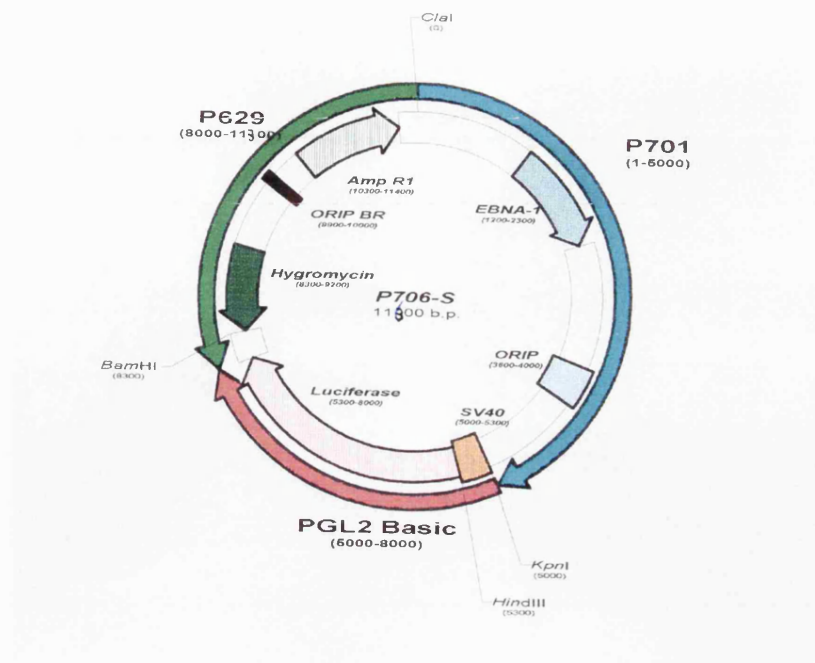




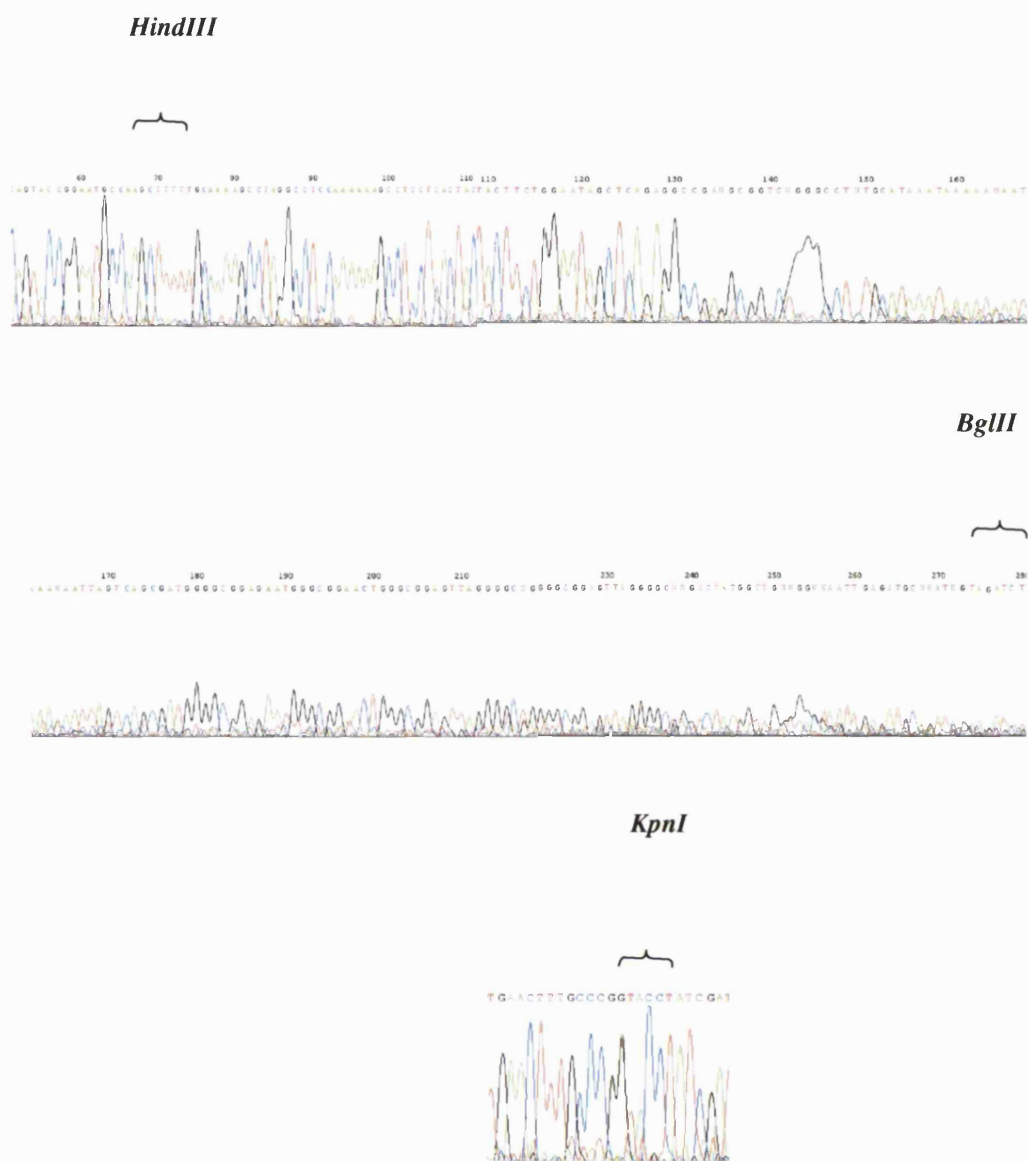
**Map 2.** p706/HNF-4



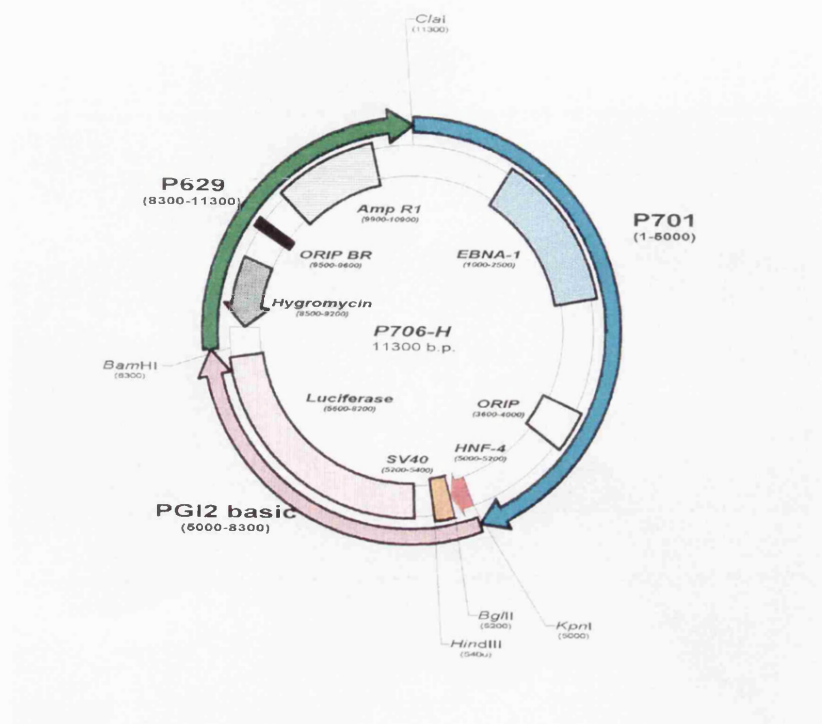
**Figure 4.1.** Confirmation of the insertion of HNF-4 repeats between *Kpn*I and *Bgl*III cloning sites of p706 DNA plasmid by sequence analysis.



**Map 3.** p706/SV40.



**Figure 4.2.** Confirmation of the insertion of HNF-4 1' and SV40 between *KpnI* and *Hind III* restriction enzymes of p706 vector by sequence analysis..



**Map 4.** p706/HNF-4/SV40.

To reinforce the time course data obtained with the luciferase activity and as an internal control, in each set of experiments both cell lines were also co-transfected in parallel with CMV- $\beta$ gal and 48 h later both the luciferase and  $\beta$ -galactosidase activities were measured in each sample. The reason for choosing the 48 h time point for  $\beta$ -galactosidase activity was that during a series of initial experiments, it was found that the maximum peak of this enzymatic activity within the transient transfected cells was around 48 h after which the  $\beta$ -galactosidase activity dropped rapidly to the background level (i.e. within the following 24 h, data not shown). Therefore for normalization and as an internal control for possible differences in the transfection efficiency, the luciferase activity (i.e. RLU/mg protein) of the 48 h time point was divided by  $\beta$ -gal activity (i.e.  $\beta$ -gal/mg protein) and expressed as RLU/ $\beta$ -gal.

#### ***4.1. The Transient Expression of p706/HNF4/SV40 Plasmids***

The first approach was to determine the transient basal level expression of luciferase gene following transfection of HepG2 cells with the p706/HNF-4/SV40 plasmids. The methodology involved the transfection of the cells with p706/HNF4/SV40 plasmids, followed by lysis of the cells 10, 24, 48, 72, 96 and 120 h following transfection assays. The level of luciferase activity was then measured in the cells and expressed as RLU/mg protein.

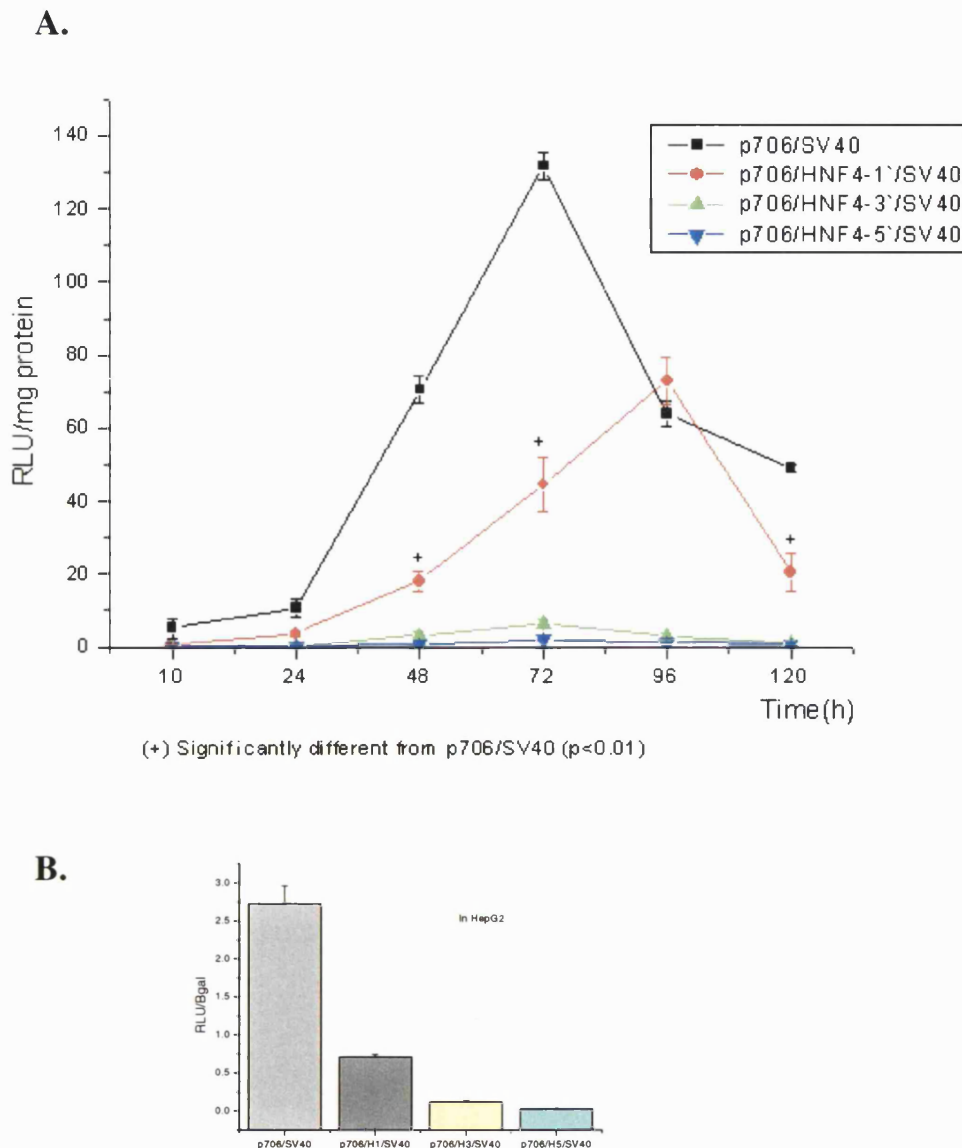
**Figure 4.1.1.A** summarizes the time course studies of the luciferase activity in HepG2 cells transfected with either p706/SV40 or p706/HNF4-1'/SV40, p706/HNF4-3'/SV40 and p706/HNF4-5'/SV40 plasmids. The results showed that the presence HNF-4 elements appear to repress the basal level of luciferase activity in HepG2 cells. Indeed the level of luciferase activity in cells transfected with p706/HNF4-1'/SV40 was two to three fold lower than that of p706/SV40 alone. The luciferase activity dropped even more when HepG2 cells were transfected with p706/HNF4-3'/SV40. The transfection of HepG2 cells with p706/HNF4-5'/SV40 revealed no basal luciferase activity when compared to p706/SV40-transfected cells.

**Figure 4.1.2.A** summarizes the time course studies of the luciferase activity in HtTA-1 cells transfected with either p706/SV40 or p706/HNF4-1'/SV40, p706/HNF4-3'/SV40 and p706/HNF4-5'/SV40 plasmids. The results showed that the presence HNF-4 elements also repress the basal level of luciferase activity in these cells, although to much lesser extent than in HepG2-transfected cells. The normalization of luciferase activity to  $\beta$ -galactosidase at 48 h time point showed similar data to those normalized per mg protein, indicating that both normalization methods can be used for the interpretation of the data obtained (**Figure 4.1.1.B. and 4.1.2.B**).

Overall, it appeared that the presence of HNF-4 elements upstream of SV40 represses significantly the basal level of luciferase activity in both cell lines. However the comparison of the level of luciferase activity in cells transfected with p706/SV40 revealed that this level was significantly higher in HepG2 cells when compared to

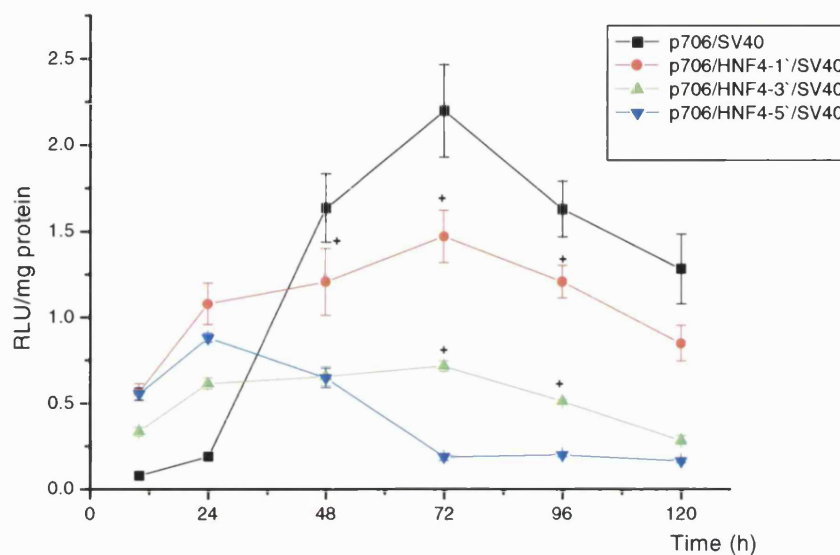
HtTA-1, suggesting that SV40 promoter of pGL3 should contain *cis*-acting elements that are liver-specific. Indeed the computer analysis using the TFSEARCH program (see **Appendix 4.1.1**) revealed that this promoter contains an element that resembles the liver-enriched transcription factor-binding site for C/EBP. The C/EBP is a transcription factor that is highly enriched in the liver and is known to regulate the expression of liver-specific genes such as albumin and the cytochrome-P450 (CYP2D5) (Kel *et al.*, 2001). Thus the C/EBP may, at least in part, regulate the expression of the luciferase gene in HepG2 cells.





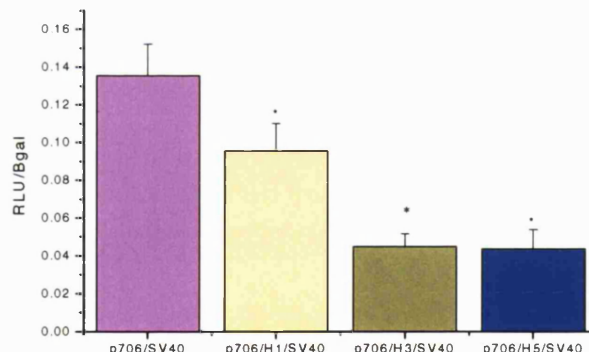
**Figure 4.1.1. Functional analysis of liver-enriched HNF-4 regulatory elements in transiently transfected HepG2 cells.** **A.** The time course of basal level of luciferase activity following transient transfection of HepG2 cells with the p706/SV40 and p706/HNF4-1'/SV40, P706/HNF4-3'/SV40 and P706/HNF4-5'/SV40 reporter plasmids. The transfected cells were kept in the tissue culture media with hygromycin. The luciferase activity was measured 10, 24, 48, 72, 96 and 120 h following transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HepG2 cells with the p706/SV40 and p706/HNF4/SV40 constructs and CMVβ-gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to β-galactosidase activity and expressed as RLU/β-gal. Data are means  $\pm$  S.D. of 4 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.05$ ) of each data point.

**A.**



(+) Significantly different from p706/SV40 ( $P < 0.01$ )

**B.**



(\*) Significantly different from p706/SV40 ( $P < 0.05$ )

**Figure 4.1.2. Functional analysis of liver-enriched HNF-4 regulatory elements in transiently transfected HtTA-1 cells.** **A.** The time course of basal level of luciferase activity following transient transfection of HtTA-1 cells with the p706/SV40 and p706/HNF4-1'/SV40, p706/HNF4-3'/SV40 and p706/HNF4-5'/SV40 reporter plasmids. The transfected cells were kept in the tissue culture media with hygromycin. The luciferase activity was measured 10, 24, 48, 72, 96 and 120 h following transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HtTA-1 cells with the p706/SV40 and p706/HNF4/SV40 constructs and CMV $\beta$ -gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to  $\beta$ -galactosidase activity and expressed as RLU/ $\beta$ -gal. Data are means  $\pm$  S.D. of 4

independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.05$ ) of each data point.

**Appendix 4.1.1: Sequence of 230 bp of the SV40 promoter of the PGL-3 plasmid.** The DNA sequences that resemble the transcription factor elements are highlighted in yellow.

```

5'                                     AP-1
GCTCGAGATCTGCGATCTGCATCTCAATTAGTCAGCAACCATAGTCC
      StRE
CGC CCCTAACTCCGCCCATCCCG CCCCTAACTC CGCCAGTTC CGCCATTCT
      AP-1
CCGCCCCATC GCTGACTAATTTTTTTTATT TATGCAGAGG CCGAGGCCGC CTCGGCCTCT
GAGCTATTCC AGAAGTAGTGAGGAGGCTTT TTTGGAGGCC
      C/EBP      HSF
AGGCTTTTGCAAAAAGCTT GGCATTCCGG
                                     3'

```

## CHAPTER 5    Result 3

The expression of HO-1 is known to be particularly high in the liver (Maines, 1988), presumably because this gene contains a series of liver-specific regulatory elements within its 5'-flanking promoter region that contribute to its higher basal level expression. The purpose of this section was to identify such potential upstream promoter regions containing key *cis*-acting elements that regulate the tissue-preferential basal expression of the HO-1 gene. To this regard, the p706-EBV-based luciferase reporter plasmids that contained progressive deletions within the 4.7 kb upstream promoter region of the human HO-1 gene were transiently transfected into two human carcinoma cell lines, HepG2 (liver-specific cells) and HtTA1 (Hela derived non-liver cells) and then the level of luciferase expression was monitored within the lysates of transfected cells. Since EBV-based episomal vectors have the advantage of autonomous replication and their expression can be maintained for several days under hygromycin, the basal level expression of luciferase in these constructs was followed up to 5 days following transient transfection. A database program TFSEARCH was then used to locate the transcription factor elements within the 4.7 kb of the 5'-flanking region of human HO-1.

### ***5.1. The Transient Expression of p706/HO4.7 Plasmid***

The first approach was to determine the transient basal level expression of luciferase gene under the control of the 4.7 kb of 5'-flanking region of the human HO-1 gene following transfection of both HepG2 and HtTA1 cells with the p706 /HO4.7 plasmid (16 kb). The methodology involved the transfection of the cells with p706/HO4.7,

followed by lysis of the cells 10, 24, 48, 72, 96 and 120 h following transfection assays. Furthermore a comparison was made between the transient basal level expression of luciferase of cells maintained either with or without hygromycin. The level of luciferase activity was then measured in the cells under both conditions and expressed as RLU/mg protein.

**Figure 5.1.1.** summarizes the time course studies of the luciferase activity in both HepG2 and HtTA-1 cells. As shown the basal level of luciferase activity in HtTA-1 cells did not significantly change till 120 h following transient transfection. However in comparison, the level of this enzymatic activity was significantly increased in HepG2 cells especially from 48 h up to 120 h post-transfection time points. This sustained increase in luciferase activity in the transfected HepG2 cells was observed under both Hygromycin-selected (**Figure 5.1.1.C**) and unselected conditions (**Figure 5.1.1.A**). However the addition of hygromycin as the selection marker significantly decreased the level of RLU/mg protein in both transfected HepG2 and HtTA-1 cells, indicating that omitting the selection marker could affect the evaluation of the data obtained.

The fact that the basal level of luciferase activity was significantly higher in liver-specific HepG2 cells when compared to the non-liver HtTA-1 cells was consistent with the hypothesis that the 4.7 kb upstream promoter region of HO-1 should contain many key liver-specific *cis*-acting regulatory elements. However to ascertain that such difference in basal level expression of luciferase was not due to variability in transfection efficiencies between the two cell lines, we performed the following control experiments:

- To reinforce the time course data obtained with the luciferase activity and as an internal control, in each set of experiments both cell lines were also co-

transfected in parallel with CMV- $\beta$ gal and 48 h later both the luciferase and  $\beta$ -galactosidase activities were measured in each sample. The reason for choosing the 48 h time point for  $\beta$ -galactosidase activity was that during a series of initial experiments, it was found that the maximum peak of this enzymatic activity within the transient transfected cells was around 48 h after which the  $\beta$ -galactosidase activity dropped rapidly to the background level (i.e. within the following 24 h, data not shown). Therefore for normalization and as an internal control for possible differences in the transfection efficiency, the luciferase activity (i.e. RLU/mg protein) of the 48 h time point was divided by  $\beta$ -gal activity (i.e.  $\beta$ -gal/mg protein) and expressed as RLU/ $\beta$ -gal.

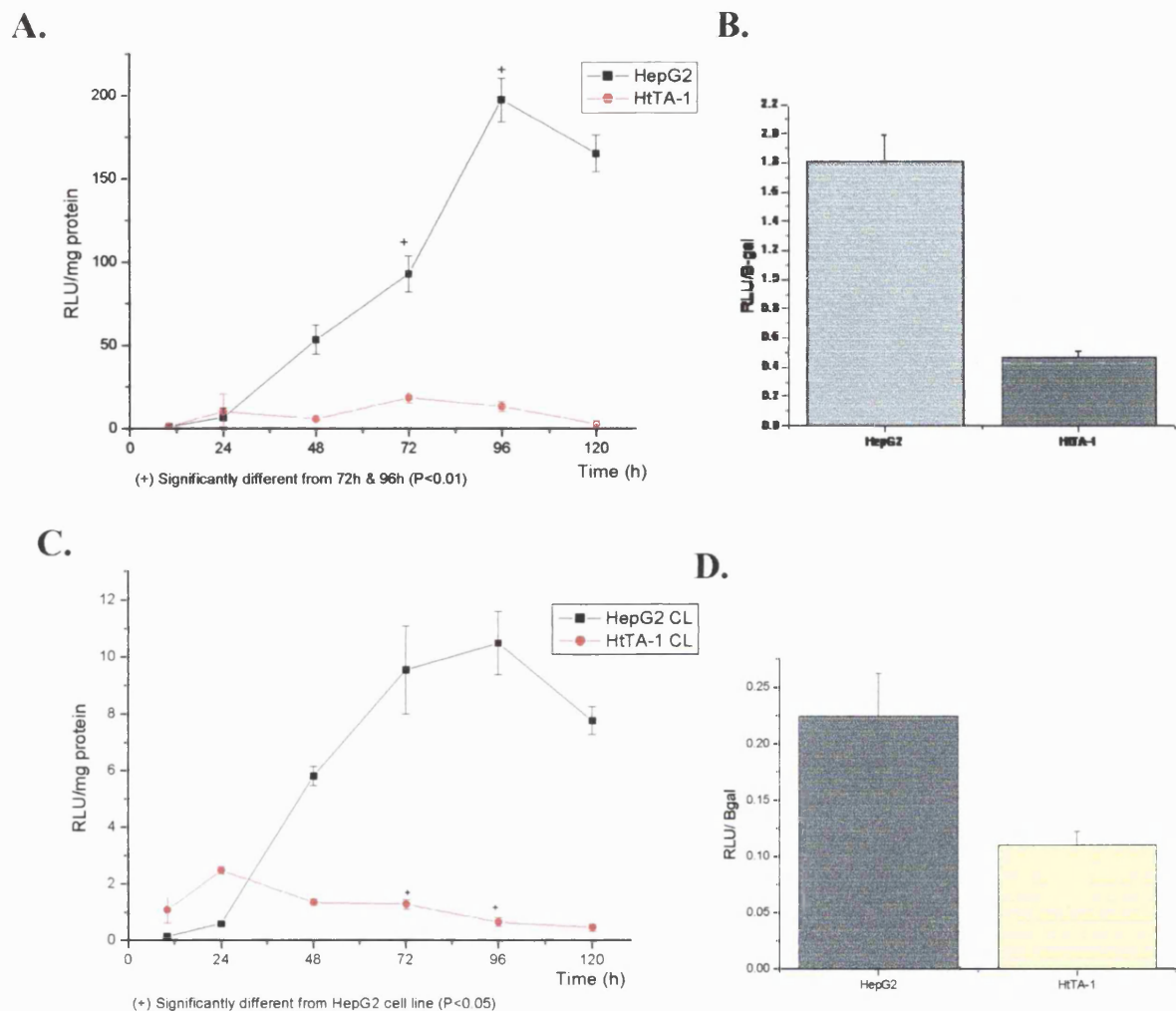
The normalization of luciferase activity to  $\beta$ -galactosidase at 48 h time point showed similar data to those normalized per mg protein, indicating that both normalization methods can be used for the interpretation of the data obtained (**Figure 5.1.1.B., 5.1.1.D. and 5.1.2.A)**

- Moreover for each experiment a series of additional controls were used such as mock-transfected cells (i.e. cells that were undergone the transfection procedure using DNA-free transfection mixtures), CMV $\beta$ -gal-transfected cells alone, plus the transfection of cells with an independent reporter plasmid called pEGFP that expresses the green fluorescent protein under control of CMV promoter. For most of these controls, the data were analysed 48 h following the transfection assays. (see **Figure 5.1.2.**).

**Figure 5.1.2.** shows representative examples of these control experiments performed 48 h following the transient transfection assays. The comparison of the level of  $\beta$ -gal activity in these two cell lines indicated that the transfection efficiency of these two cell

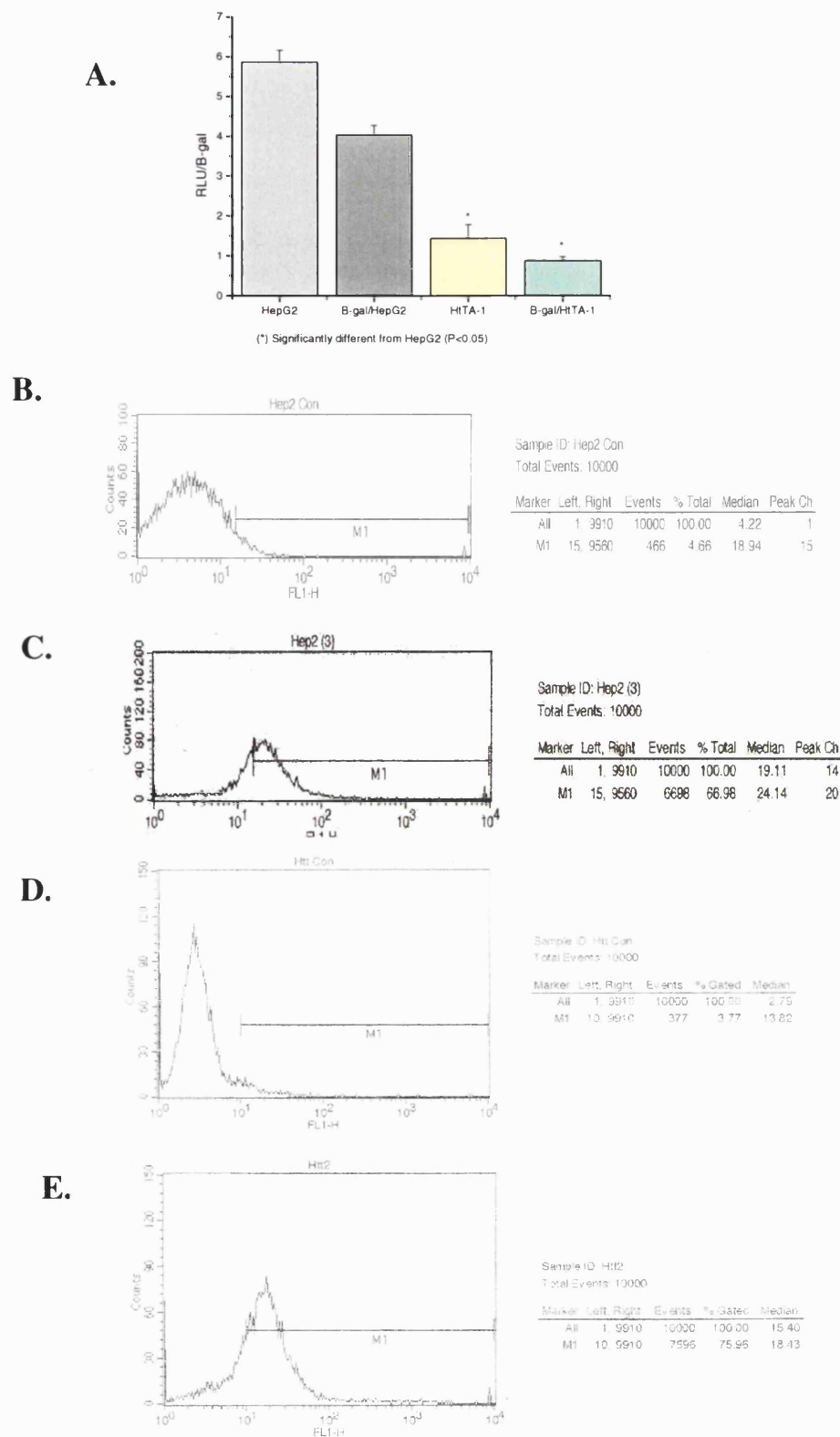
lines are in the same range of 18 to 21 as a result the level of normalised luciferase activity is always higher in HepG2 cells than HtTA-1 cells (**Figure 5.1.2.A**). Similarly, when these two cell lines were transfected with an independent reporter plasmid (i.e. pEGFP) the estimated percentage of transfection efficiency in flow cytometry (i.e. the observed shift in fluorescence with respect to X-axis) was in the very close range of 60% to 70%. The fluorograms of a representative expression are included in **Figure 5.1.2. B to 5.1.2.E**.

This set of data is consistent with the notion that the differences in basal level expression of luciferase in cells transfected with p706/HO4.7 are not due to variability in transfection efficiencies between the two cell lines, but rather related to the tissue/cell-preferential expression of this enzyme that functions under the control of liver-specific *cis*-regulatory elements present within the 5'-flanking promoter region of HO-1 gene.



**Figure 5.1.1. Functional analysis of 4.7 kb of human HO-1 5'-flanking upstream promoter region in transient transfection assays.** **A.** The time course of basal level of luciferase activity following transient transfection of HepG2 (black square) and HtTA-1 (red circle) cells with the p706/HO4.7 reporter plasmid. The transfected cells were kept in the tissue culture media without hygromycin. The luciferase activity was measured 10, 24, 48, 72, 96 and 120 h following transient transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HepG2 and HtTA-1 cells with the p706/HO4.7 and CMVβ-gal reporter plasmids. The transfected cells were kept in the media without hygromycin. The luciferase activity was normalised to β-galactosidase activity and expressed as RLU/β-gal. **C.** The time course of basal level of luciferase activity following transient transfection of HepG2 (black square) and HtTA-1 (red circle) cells with the p706/HO4.7 reporter plasmid. The transfection procedure and time points were similar to (A) except that the transfected cells were incubated in the media containing hygromycin. **D.** The normalised basal luciferase activity measured 48 h following transient co-transfection of HepG2 and HtTA-1 cells with the p706/HO4.7 and CMVβ-gal reporter plasmids. The transfection procedure and normalization of the data was similar to (B) except that the transfected cells were incubated in the media containing hygromycin. Data are means  $\pm$  S.D. of 3 to 4 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.05$ ) of each data points.





**Figure 5.1.2. Comparison of the transfection efficiency between HepG2 and HtTa-1 cell lines.** **A.** The basal level of luciferase activity 48 h following transient transfection of HepG2 and HtTA-1 cells with either the p706/HO4.7 reporter plasmid alone or combined with CMV- $\beta$ -gal reporter plasmid. The data are means  $\pm$  S.D. of 3 to 4 independent experiments. **B.** The fluorogram showing the background fluorescence of mock-transfected HepG2 cells. The X-axis shows the fluorescence intensity and the Y-axis is the cell counts **C.** The fluorogram of the expression of GFP 48 h after transient transfection of HepG2 cells with pEGFP plasmid. **D.** The background fluorescence of mock-transfected HtTA-1 cells. **E.** The fluorogram of the expression of GFP 48 h following transient transfection of HtTA-1 cells with pEGFP plasmid.

## ***5.2. The Transient Expression of p706/HO Sequence Deletion-Mutant Plasmids***

The p706/HO4.7 and its sequence-deletion mutant plasmids were originally constructed by Larry Richman in the laboratory of Prof. Tyrrell (Pharmacy and Pharmacology Department) to identify the UVA-responsive elements present within the 5'-flanking region of the HO-1 gene. Since the Dnase I hypersensitive (HS) sites within the mammalian chromatin are often located within the 5'-flanking region of the active genes and are usually associated with functional sequences such as an 'enhancer', 'silencer' or 'promoter', Tyrrell and co-workers determined the HS sites within the 4.7 kb of 5'-flanking region of the human HO-1 gene using the method developed by Wu *et al.*, (1979). These investigations (unpublished data) resulted in the identification of three HS sites called HS-1 (approximate position from -70 to +30), HS-2 (approximate position -280 to -180) and HS-3 (approximate position -3650 to -4080). The HS-3 site overlapped with a region homologous to the mouse enhancer elements (E1). The p706/HO sequence deletion mutants were prepared by partial or complete deletion of the HS sites and their surrounding regions.

**Figure 5.2.1.** shows a diagrammatic representation of the HS sites mapped within the 4.7 kb of 5'-flanking region of the human HO-1 and the sequence-deletion analysis performed on p706-derived vectors.

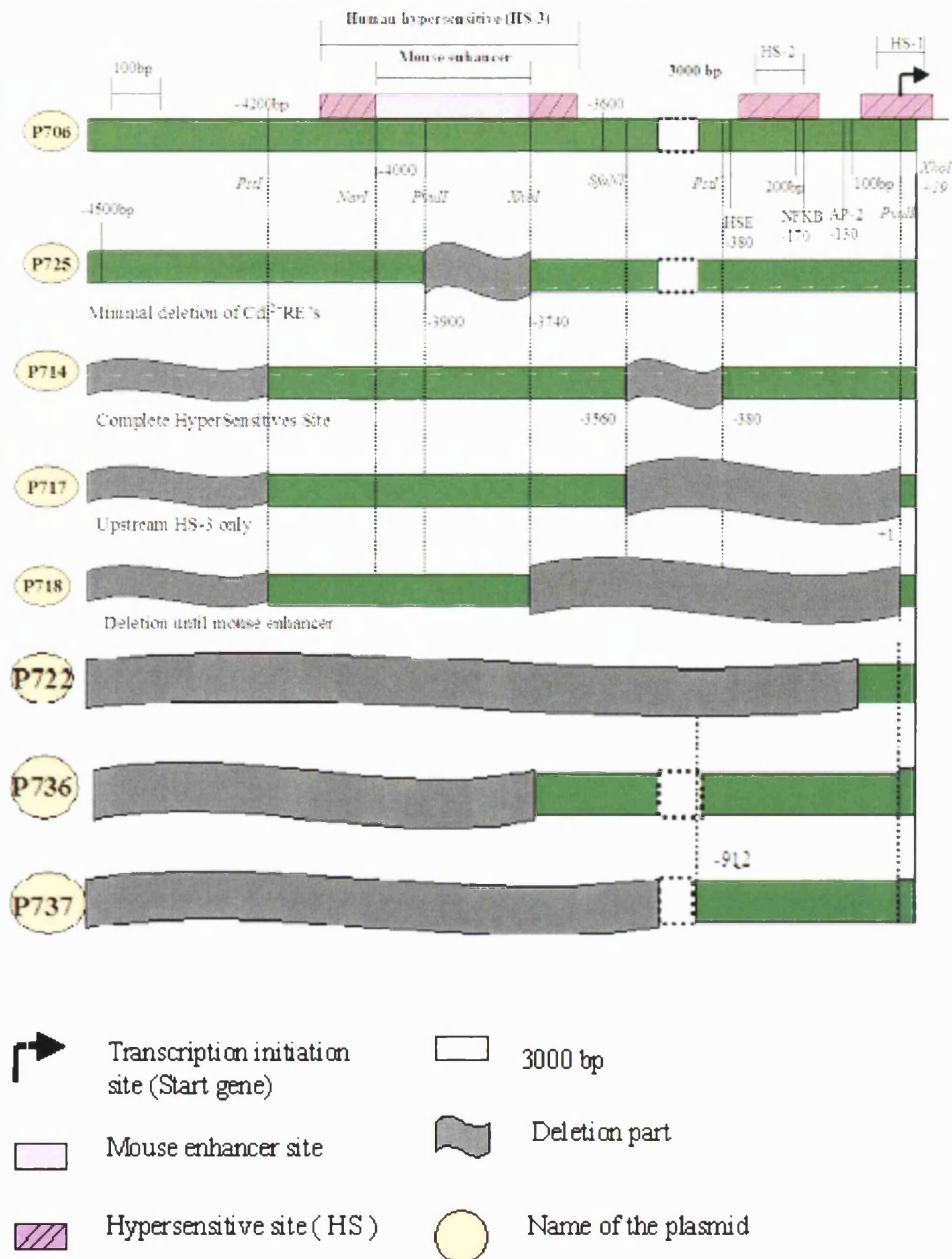
A brief description of these sequence-deletion mutant plasmids is outlined below:

- **p706 (i.e. p706/HO4.7)** contains the full 4.7 kb of the 5'-flanking region of the human HO-1 until plus 30 bp downstream of the transcription initiation site i.e. from nucleotide position -4740 to +19 so this construct contains the HS-1, HS-2 and HS-3 sites.
- **p725** has a minimal deletion in cadmium responses element ( $\text{Cd}^{+2}\text{RE}$ ) from nucleotide position -3900 to -3740. This region overlaps with functional parts of HS-3 and minimal mouse enhancer region SX2.
- **p714** contains the complete HS sites but has deletions in two regions: (1) Deletion from nucleotide position -3560 to -380; (2) Deletion from nucleotide position -4740 to -4200.
- **p717** contains only the HS-3 site and has deletions in two major regions: (1) Deletion from nucleotide position -3560 to +1, which contains the proximal promoter region as well as the complete HS-2 and the partial HS-1 sites; (2) Deletion from nucleotide position -4740 to -4200.
- **p718** contains the intact mouse enhancer region but has deletions in two major regions: (1) Deletion from nucleotide position -3740 to +1, which contains the proximal promoter region, the complete HS-2 and partial HS-1 sites plus a

partial deletion of HS-3 site until the mouse enhancer region; (2) Deletion from nucleotide position -4740 to -4200.

- **p722** has a major deletion of the full 4.7 kb 5'-flanking promoter region from positions -4740 to +1 and contains only the partial sequence of the HS-1 i.e. position +1 to +19.
- **p736** contains the intact HS-2 site and has deletions in two regions: (1) Partial deletion of HS-1 site from nucleotide position +19 to +30; (2) Deletion from position -4740 to -3740 that contains most of the HS-3 site plus the entire mouse enhancer region containing the Cd<sup>+2</sup>RE and its upstream region.
- **p737** contains the intact HS-2 site and has deletions in two regions: (1) Partial deletion of HS-1 site from nucleotide position +19 to +30; (2) Deletion from nucleotide position -4740 to -912 that contains deletions of HS-3 site plus the entire mouse enhancer site containing the Cd<sup>+2</sup>RE and its upstream region.

### HO-1 promoter deletion analysis on EBV vectors



**Figure 5.2.1.** A diagrammatic representation of the HS and partial restriction-endonucleases sites mapped within the 4.7 kb of 5'-flanking region of the human HO-1 and its sequence-deletion analysis on p706-derived vectors.

To identify the potential upstream promoter regions of HO-1 containing the *cis*-acting elements that contribute to tissue-preferential expression of HO-1 gene, both HepG2 and HtTA-1 cell lines were transfected with p706/HO sequence deletion mutant reporter plasmids. The latter reporter plasmids contained a series of sequence deletions within the 4.7 kb of the 5'-flanking promoter region of human HO-1 gene. The luciferase activity was measured 10, 24, 48, 72, 96 and 120 h following transfection assays and expressed as RLU/mg protein. To reinforce the time course data obtained with the luciferase activity and as an internal control, in each set of experiments both cell lines were also co-transfected in parallel with CMV- $\beta$ gal and 48 h later the luciferase activity was normalised to  $\beta$ -galactosidase activity and expressed as RLU/ $\beta$ -gal.

**Figure 5.2.2** to **Figure 5.2.5.** summarise the data obtained from these transfection assays in both HepG2 and HtTA-1 cell lines, respectively. Some of the main observations are outlined below:

- (1) The first observation was that the basal level of luciferase activity of the mutant reporter plasmids in HepG2-transfected cells was overall higher than in HtTA-1-transfected cells.
- (2) Secondly, as shown in **Figure 5.2.2.A.**, in HepG2 cells, the 260 bp Cd<sup>2+</sup>RE deletion from nucleotide position -3900 to -3740 in p725 reporter plasmid resulted in a significant reduction (i.e. 3 to 6 fold reduction) of the promoter activity when compared to that of p706/HO4.7 plasmid. This reduction was also observed in HtTA-1 cells (**Figure 5.2.3.A.**) although to a lesser extent (i.e. 2 to 4 fold reduction).

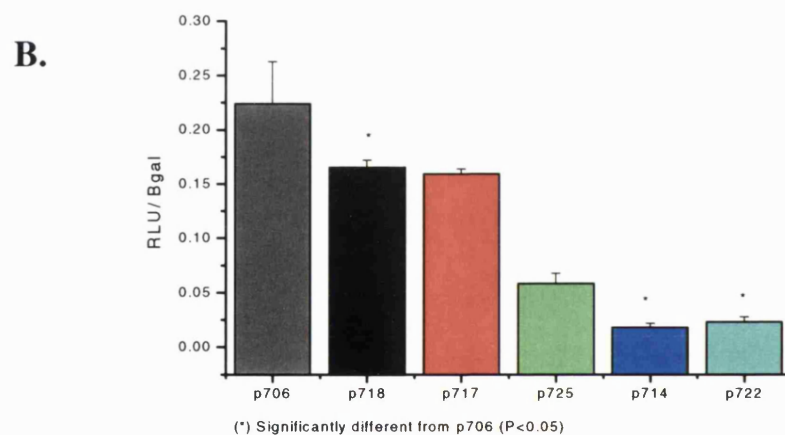
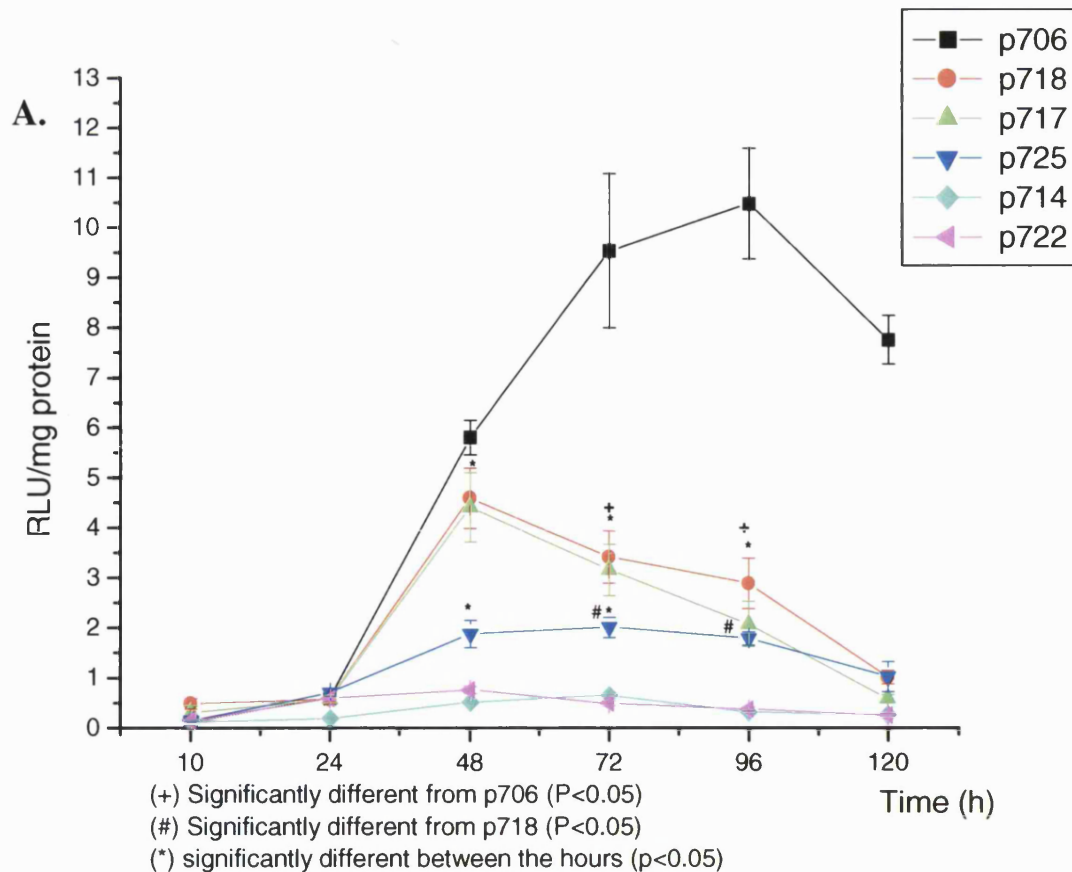
- (3) As shown in **Figure 5.2.2.A.**, the basal level of luciferase activity in HepG2 cells transfected with p717 and p718 was similar and significantly higher than those cells transfected with p725 although the former mutant constructs contained major deletions within the 4.7 kb upstream promoter region i.e. A common deletion from nucleotide positions –4740 to –4200 and then different deletions in p717 from nucleotide position –3560 to +1 and in p718 from nucleotide position –3740 to +1. These data suggest that firstly the region –3740 to –3560 does not contain any significant element responsible for basal promoter activity and that secondly the HS-3/ mouse enhancer/ Cd<sup>+2</sup>RE region must contain positive liver-preferential regulatory elements as the basal activity of luciferase in HtTA-1 cells transfected with p717 and p718 is very low and similar to that of p725 (**Figure 5.2.3.A.**).
- (4) Interestingly, the basal level of luciferase activity in HepG2 cells transfected with p714 plasmid was extremely low and comparable to that of p722 that contains the complete deletion of the 4.7 kb upstream promoter region of the human HO-1 gene. The comparison of p714 mutant construct with p717 and p718 strongly suggest that the region –380 to +1 should contain liver-preferential negative regulatory elements, as the transient expression of luciferase in HtTA-1 cells transfected with p714 was relatively high and almost similar to that of p706 (**Figure 5.2.2.A.** and **Figure 5.2.3.A.**).
- (5) The basal level of luciferase activity in HepG2 cells transfected with p737 was relatively high and similar to the activity observed with p717 and p718. The

comparison of the expression of luciferase activity in HepG2 and HtTA-1 cells transfected with this reporter plasmid suggests that the proximal promoter of the HO-1 from nucleotide position –912 to +1 should contain liver-preferential positive regulatory elements (**Figure 5.2.2.A.** and **Figure 5.2.4.A.**).

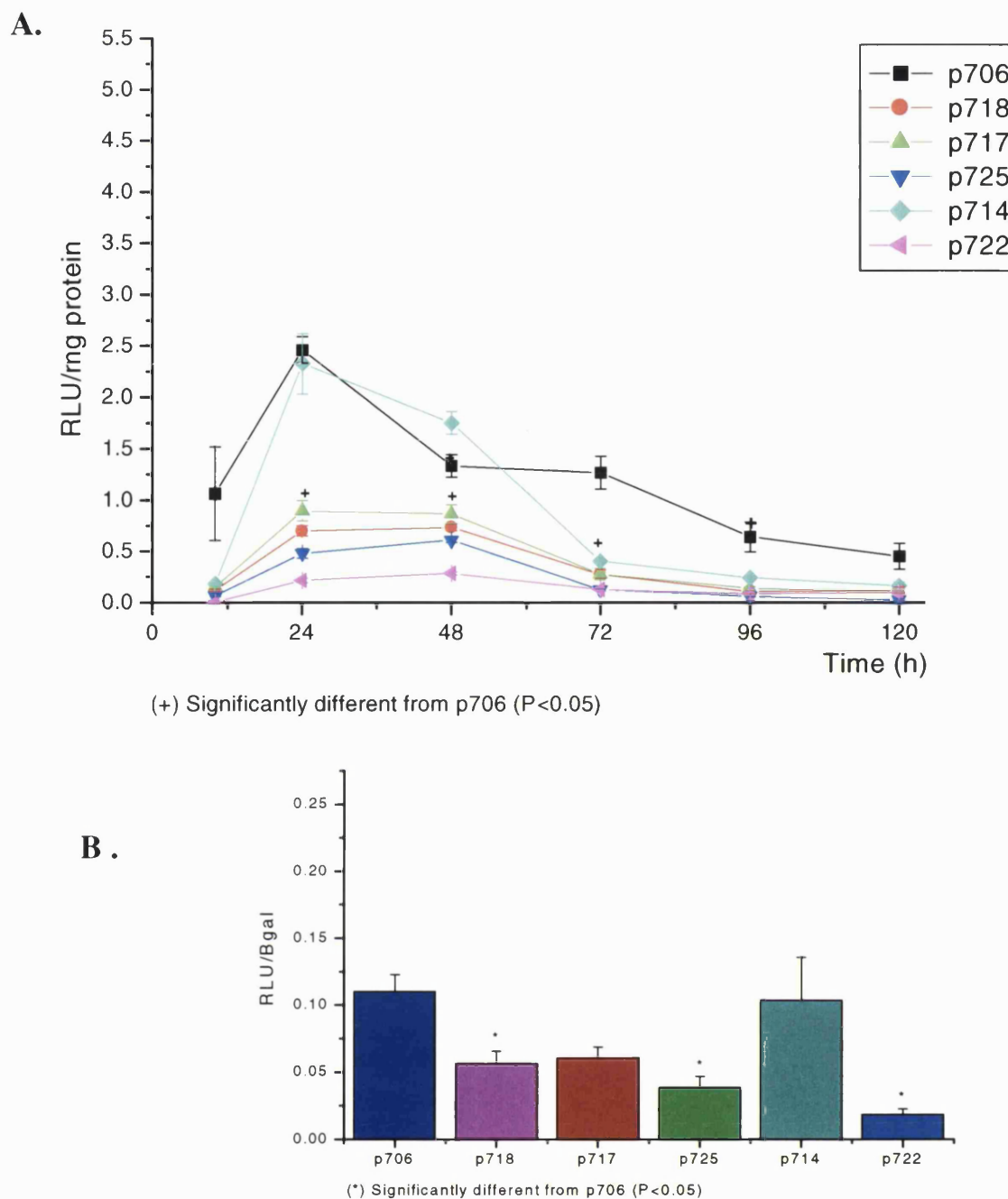
- (6) Finally the basal level of luciferase activity in HepG2 cells transfected with p736 was the highest activity observed among the p706 sequence deletion mutants, strongly indicating that the region –3740 to –912 should contain most of the positive regulatory elements for liver-preferential expression of HO-1 gene. Indeed the transfection of this reporter plasmid in HtTA-1 cells did not modify the low basal level of luciferase activity in these cells (**Figure 5.2.4.A.** and **Figure 5.2.5.A.**).

In all these transfection assays the data normalised by  $\beta$ -gal (i.e. 48 h post-transfection time, **Figure 5.2.4.B.** to **Figure 5.2.5.B.**) were similar to the data normalised to mg protein.

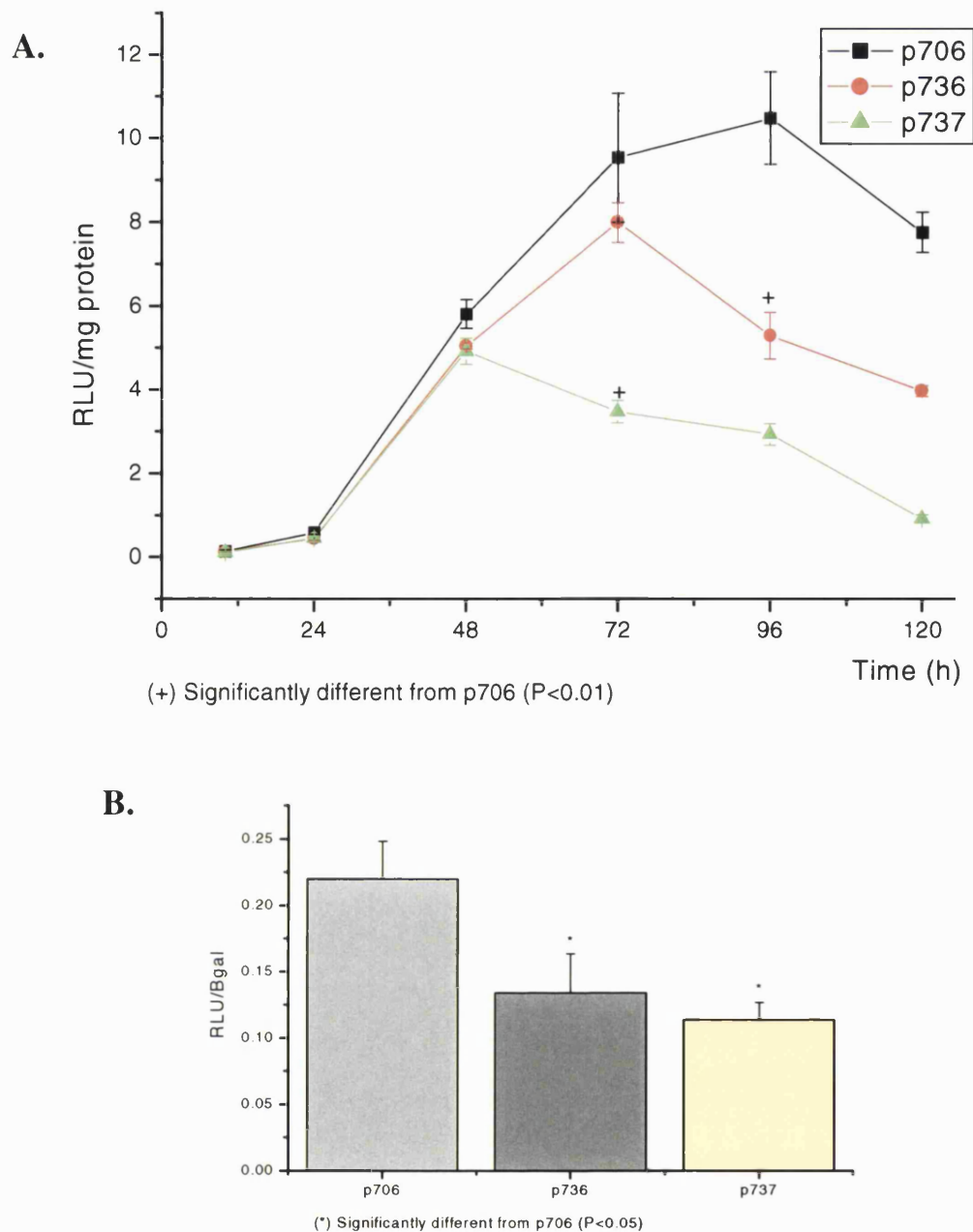




**Figure 5.2.2. Functional analysis of human HO-1 5'-flanking sequence-deletion mutants p714, p717, p718, p722 and p725 in transiently transfected HepG2 cells.** **A.** The time course of basal level of luciferase activity following transient transfection of HepG2 cells with the p706/HO4.7 and p706/HO-sequence deletion mutant reporter plasmids. The transfected cells were kept in the tissue culture media with hygromycin. The luciferase activity was measured following transfection assays expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HepG2 cells with the p706/HO mutants and CMVβ-gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to β-galactosidase activity and expressed as RLU/β-gal. Data are means  $\pm$  S.D. of 3 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.05$ ) of each data point.

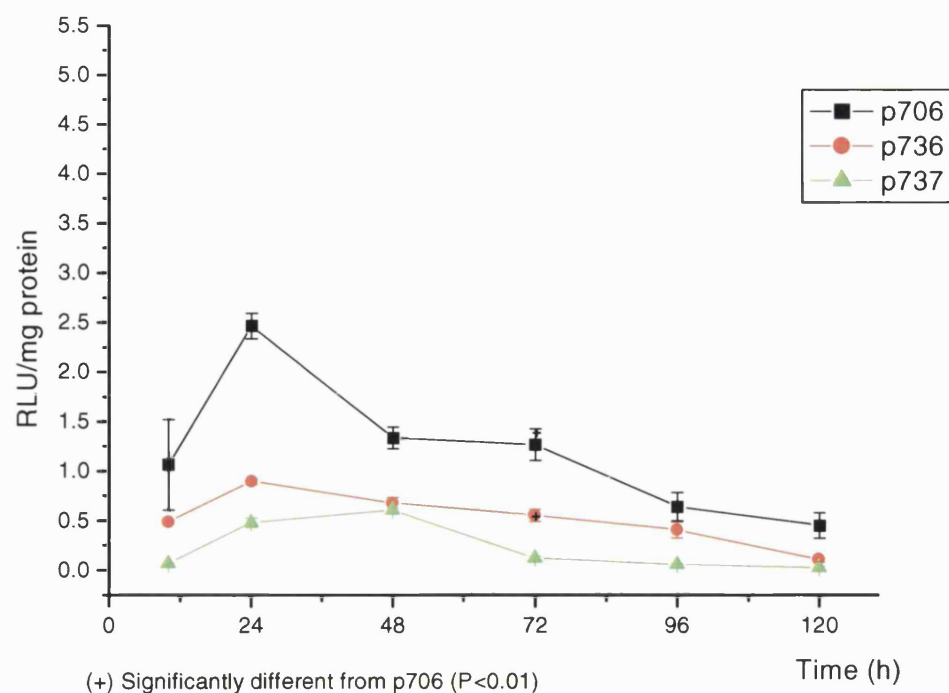


**Figure 5.2.3. Functional analysis of human HO-1 5'-flanking sequence-deletion mutants P714, P717, P718, P722 and P725 in transiently transfected HtTA-1 cells.** **A.** The time course of the basal level of luciferase activity following transient transfection of HtTA-1 cells with the p706/HO4.7 and P706/HO-sequence deletion mutant reporter plasmids. The transfected cells were kept in the tissue culture media with hygromycin. The luciferase activity was measured following transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HtTA-1 cells with the P706/HO mutants and CMV $\beta$ -gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to  $\beta$ -galactosidase activity and expressed as RLU/ $\beta$ -gal. Data are means  $\pm$  S.D. of 4 to 5 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.05$ ) of each data point.

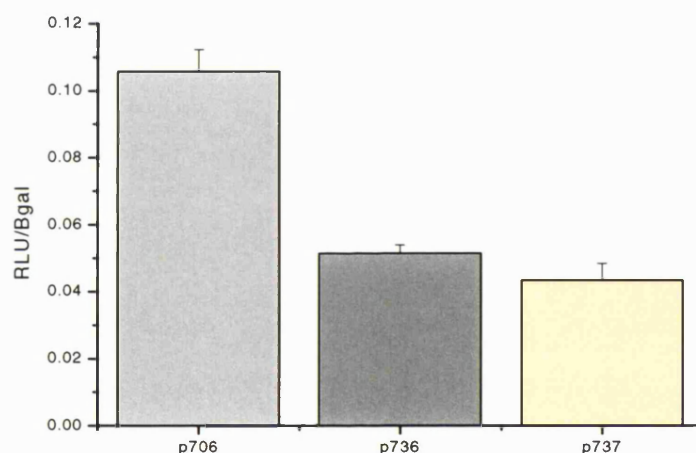


**Figure 5.2.4. Functional analysis of human HO-1 5'-flanking sequence-deletion mutants p736 and p737 in transiently transfected HepG2 cells.** **A.** The time course of basal level of luciferase activity following transient transfection of HepG2 cells with the p706/HO4.7 and p706/HO-sequence deletion mutant reporter plasmids in the presence of hygromycin. The luciferase activity was measured following transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HepG2 cells with the p706/HO mutants and CMV $\beta$ -gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to  $\beta$ -galactosidase activity and expressed as RLU/ $\beta$ -gal. Data are means  $\pm$  S.D. of 4 to 5 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.01$ ) of each data point.

**A.**



**B.**



**Figure 5.2.5. Functional analysis of human HO-1 5'-flanking sequence-deletion mutants p736 and p737 in transient transfected HtTA-1 cells.** **A.** The time course of basal level of luciferase activity following transient transfection of HtTA-1 cells with the p706/HO4.7 and p706/HO-sequence deletion mutant reporter plasmids in presence of hygromycin. The luciferase activity was measured following transfection assays and expressed as RLU/mg protein. **B.** The normalised basal luciferase activity measured 48 h following co-transfection of HtTA-1 cells with the p706/HO mutants and CMVβ-gal reporter plasmids. The transfected cells were kept in the media with hygromycin. The luciferase activity was normalised to β-galactosidase activity and expressed as RLU/β-gal. Data are means  $\pm$  S.D. of 3 to 4 independent experiments. The Student *t*-test was used to evaluate the statistical significance ( $p < 0.01$ ) of each data point.

### ***5.3. Computer Analysis of the Regulatory Regions of the 5'-Flanking Region of the Human HO-1 Gene***

To identify the potential *cis*-acting elements that might play a role in the modulation of the basal level of luciferase activity in transfection assays involving the sequence-deletion mutant reporter plasmids, a TFSEARCH computer program was used to locate the regulatory elements within the 4.7 kb of the 5'-flanking region of the human HO-1 gene. Furthermore because of the extensive research data available from studies carried out in the mouse HO-1 gene, the TFSEARCH was also performed within the 4.7 kb of the 5'-flanking in the mouse HO-1 gene to compare the potential regulatory elements between the 4.7 kb of 5'-flanking region of human and murine HO-1 gene. The **Appendix 5.3.1** and **Appendix 5.3.2** summarises the main findings of the TFSEARCH analysis for both human and mouse HO-1 gene, respectively. The *cis*-regulatory elements that have been previously identified and published are shown in bold characters. The computer research of the present study has also revealed numerous new binding sites for several known transcription factors, although within the appendix, only the most relevant ones are indicated.

The comparison of the TFSEARCH data and the sequence-deletion mutant transfection assays suggest some *cis*-acting elements as potential candidate for liver-preferential expression of human HO-1 gene. A summary of this comparative study is outlined below:

- As mentioned previously the Cd<sup>+2</sup>RE region (from nucleotide position –3740 to –3900) appears to contain liver-preferential positive regulatory elements. This

fragment indeed contains the C/EBP $\beta$  site. The C/EBP $\beta$  is a transcription factor that is highly enriched in the liver and is known to regulate the expression of liver-specific genes such as albumin and the cytochrome-P450 (CYP2D5) (Kel *et al.*, 2001). Thus the C/EBP $\beta$  may, at least in part, regulate the expression of the HO-1 gene in HepG2 cells.

- Also the fact that the basal level of luciferase activity in HepG2 cells transfected with p717 and p718 was similar and significantly higher than those cells transfected with p725 further suggested that the region –4200 to –3740 should contain positive liver-preferential regulatory elements. The computer analysis revealed that in addition to the C/EBP $\beta$  site present within the Cd<sup>+2</sup>RE, the region –4200 to –3740 contains a second C/EBP $\beta$  site situated between nucleotide positions –4058 and –4045. Moreover this region is rich in critical regulatory elements such as AP-1 (2 sites), AP-4, NF-E2 and StRE sites. These transcription binding sites were also present within the SV40 promoter site of pGL-3 that when inserted into p706-based EBV vector, it dramatically increased the level of luciferase activity in HepG2 cells only (see **Result 2, Figure 4.1.1.**). From these data a picture emerges suggesting that these regulatory elements might have a role in liver-preferential gene expression.
- The comparison of the luciferase activity in HepG2 cells transfected with p714 and those transfected with p717 and p718 mutant constructs strongly suggested that the region –380 to +1 should contain liver-preferential negative regulatory elements. The TFSEARCH and the published data from the literature (see **introduction section 1.2.6.1.**) reveal the presence of several critical binding sites such as AP-2 (two sites), AP-4, USF and NF- $\kappa$ B. Furthermore this region contains the highly polymorphic (GT)<sub>n</sub> repeats that are thought to act as a

repressor of the human HO-1 transcriptional activation (Kimpura *et al.*, 1997). Because of the polymorphic nature of these dinucleotide repeats and its importance in negative regulation of gene transcription (Yamada *et al.*, 2000), the number of these repeats is now carefully monitored within the promoter region of the genes. For this reason, we have also monitored these repeats within the 5'-flanking region of the p706/HO4.7 by sequencing its first 1 kb fragment. This analysis (**Appendix 5.3.3**) revealed that indeed these GT repeats (i.e. 29.repeats) are located within the proximal promoter region between nucleotide positions -197 and -258.



**Appendix 5.3.1: Sequence of 4.7 kbp of the 5'-region of the Human HO-1 gene.** The DNA sequences that resemble the transcription factor elements are highlighted in yellow. To distinguish between two attached consecutive binding elements, the highlight of one of the elements has been changed to light blue colour. The published transcription factor binding sites in highlighted yellow boxes are indicated in 'bold'. The HS sites are underlined in blue. The GT repeats within the HS-2 are indicated in 'bold'. The mouse enhancer site within the HS-3 are indicated in dark blue. The Cd<sup>2+</sup>RE sequences within the HS-3 highlighted in red.

**-4714** C/EBPb AP-4  
TTCACCCAGGCTGGAATGCAGTGGTGAATCTCAGCTCACTGCAACCTCCACCTCCCGGGTTCAAGCGAT  
AP-4  
TCTCCTGCCTCAGCTCCCCGAGTAGCTGGGATTACGGGCACCTGCCACTACGCCCGGCTAATTTTTGTA  
TTTTTAGTAGAGATGGGGTTTCACCATGTTGGCCAGGCTGGTCTTGAACCTCTGACCTCAGGTGATCCAC  
AP-4  
CCACCTCAGCTCCAGAGTGCTAGGATTACAGGCGTGAGCCACCACAATTGGCCCAGTCTATGGATTTTT  
AP-4  
AAAAAATAACTTGGGCTTGTCTTCCTTGCTGAAGAAGGCTGTTTCCAGCCTGTCACACAGCAGTTAGGC  
StRE  
TGTAGACCCTCACTGGAGCCCCCTGCCTGCAGAATCGAGCACATGTTCTTTCTGGGTCTGTGTGGGGTGT  
HNF-3b NF-kB  
GGGTGTGGTCAGGACACCGTCTGTGACCTTATTTACTGGGACGTCCCCTGCTTGAGGAGAATATCCAGG  
CAAGGTCTCCTCACTCTCCTTAGCCACCATCCAGCTTGCCCAAGAGGCCTCCCCATGATTCTCCACGC  
StRE AP-1  
CAGGGCCTCGCCTTTGTTTTTCACTGTTAGGGGCTGGCGAGTCACTGACCCGCCCCCTCCCTGCTGCCC  
AAACCACTTCTGTTTCCTGAAGG  
**-4080** 1 NF-E2 C/EBPb  
CGCCTTGGAATGCTGAGTCGCCTCGTGCAGCTGCATTCTGCTGCGTCATGTT  
Cd<sup>2+</sup>RE AP-1a  
TGGGAGGGGGGACTCGCGGAAACAAAGGGAAGCGGATTTCCTAGATTTCTGAGTCACCAGTGCCT  
AP-4  
CCTCAGCTTCTCTTAGGTGGGAGGTGAAAGGGCAGCTTTAATGGTAGGCAGGAGGAAGTGAAACTTCTA  
GAAACGGCAGAAGCCTCTTGTGTTGCTTTTCTAAGCTGTGCTTCCCCCTGCTGGGTCCCCCAGCTCC  
C/EBPb  
CACCTGTGAAGTCCACCTCAATCTGCCCTGATTAAATGTTGCAATCCACCGAGGCCCTCTTATGCT  
AP-1b  
TCACATCTCTCTGACTTCTGCACTCTGCCTCATCTCTTCTGCCTCCAGCAGGAGAAAGTTCTCTACTTTTT  
HS-3  
TTGCCTGGATAATTAT  
**-3650**  
TGTATTTTTAGTAGAGATGGGGGTTTCACCATGTTGGTCAGGCTGGTCTTGAACCTCCTGACCTCAGGTGA  
TCAGCCACCTCAGCCTCCCAAAGTGCTGGGATTATAGGTATGAGCCACCGTGCCAGCCTCATCTCCA  
TCTTCAAAGCTAGCAGCATGGCATCTCCAGTCTCT  
AP-1  
CTCTCTGTCTCTGCTTTCTTTGTACCTTCTGTATGTGCAACCATCCAGCCTCCCTCTTATAAGGCCCA  
AP-1 C/EBPb  
AGAGGATAATCCAAGATAATTCCCCATCTCAACATCCTTGACTTAATCACTCTGCAAGATCCTTTTGCT  
StRE  
ATGTAAGGTAGCAAAAAGGTAACCTATTACAGGTTCTGGTGATTAGAATGTGGACACCTTAAGGGGCT  
TTTATTTGGCCAGCAGGAGGAGAGAATAGCATTTGGATCATCTGTGCTTGACTAAAAGAAGGGG  
AP-1  
AAGTCAGGAGGCTGAATCAGCATGCGAAAGGGCCACAAATTCAGCCCTGGGACAGCCTAGAGGACCAGAA  
CAACTCTGGCCTGGCCTCTTGACCCAGCAACCCACTGTGTGGGGGCTGTTCTAACGAAGTCTGGGGC  
AP-1  
TCCAGAGAACAGTTAGAAAAGAAAGCAAGCCAGACCGGCAGCCAGGAACCTGGGTCACTGGAGGTTTC  
TCTTTCCAATGGGGGGCATAGCCTGGCCAAGTCCCTCCTGCTCTTGGGGTCCCTGCTTCCCTGTGTTAAA  
TAAAAGGGTTCAGATCTCATGAGTTTTACCCTCTAGGGTCGCTGAATGGACAGACTCTGGATCTATACA





**Continued (Appendix 5.3.1: Sequence of 4.7 kb of the 5'-region of the Human HO-1 gene).**

[illegible]

**Appendix 5.3.2: Sequence of 4.7 kb of the 5'-region of the mouse HO-1 gene.** The DNA sequences that resemble the transcription factor elements are highlighted in yellow. To distinguish between two attached consecutive binding elements, the highlight of one of the elements has been changed to light blue colour. The published transcription factor binding sites in highlighted yellow boxes are indicated in 'bold'. The CT repeats are underlined. The published enhancer elements SX2 in bold dark blue.

**-4755**

HSE  
CAAAAG**TTTTCTT**GGGGAGGCTAGCAAAGGCTGAGGATAAAAGACTTCCTGGTTCAGGGCTAGAAGTTTC  
AP-1  
TCCTGTGAGT**CTGACACATT**TGGTGTGGGGGAGGGCTGGGCCAGGTGTGCTAGGATAAGGGCACTTTACC  
StRE  
**TGAGGGGT**AGCCAAGAGCAGTTGAACGATGTTGACCAGGGGTGGGCAGCTCCCTCCATTACTGTCCTCTG  
CREB AP-4  
**AAGTCAT**TGGGCAGGAGGAAGAGAAAATGGGGATGGGGGGGGGAGGCTCCAGCTCTCTAT**CCAGCTGTC**  
AP-4  
**CAGCTGCTGTCTGTTGGAGGAACGTTCATAGGACTAACAGGGGCAGCTGTCATTTGTGCTGTCTTTTGAC**  
**TTTGAGCAGCTGTTTTATTAGGAATTCCTCTCTTTGTCTACGTGTGTGGCAGTACAGCTCTGCCTGCC**  
AP-1 AP-4  
**TGTCAGAGGGTTCTACCTTACCTCATGGATGCTTCTGTTAGTAAGATTCCCTGTCTCCATGTCAGCTGTTG**  
**AGCATTTTCTCAGCTTTGGACTGTCTGCTCTGTTGATACACAGTGCATATGTAGGCTGCAGAGCCCCACT**  
StRE  
GGAGTCTCCTGCCTTCCAGGGATAGGGACATGTC**CCCCCTC**TGGTCTGTCTCAGGCTTAGTGTAATCAGT  
ATGCTTGCTGTCTAGGAGGATTCCCTGCCTGAAGACACCCACACAAATTCCTTACTGCCATCTAACTGGG  
ACAAAAGGCACAAAGAGCTCCACCCCCACCCAGGATTCCAGCCCCACAGGAGCTGAACTTTGTTTTTCC  
AP-4 NF-E2  
**CGCAGCGGT**GGAATGCTGAGTTGTGATTTCTCACTGCTCATTTCCTCAGCTGCTTTTAT**GTCTGTGTCA**  
AP-1  
TGGTTGGGAGGGGTGATTAGCAGACAAAGGAAGACAGATTT**TGCTGAGTCACC**CTCTGTTCCCTCAGCC  
AP-4 C/EBP  
**TCAGCTAGGAATAGTTGGTAAA**AGTTCCGGAACGGCAGGCAGAGGAAGTAAGTTCTAGAAAGGCAC  
C/EBP  
AGAAGTTTCTTGTTTAAATTTTAAAT**TGTTTTGCAAAGCC**TATCTGAAACACTTCGAACTGGTACACACAC  
ACACACACACACACACACACACACACTCACACAGCGTTGCTGCTGTTCCCCCATGCCTCTCAG  
AP-1  
TTACAGAAGCTAGCAGTTTCATCCGTCTAATGGAAAGCTTTGGAGTTATG**GCTGACTCCT**TTCTTAGGTC  
TGTCCTATTCTGAAATACAGCATGGTTGTCTGTGAGATGAGCTCAGGAGTGTGGGTAGGGTTGGACACTT  
TCTAGCTTCCCTGAGGCTGCCTGTGCATCTCAGCCCTTTCGTCTGTGTCCCCCATCTTTAGAGGAGGCAG  
C/EBP  
GGTCATCCATCAACTGTGTCTGA**ACCCATCATGCAATTT**GTGATGATCCTGGATCCTGAGGGATGGGCCA  
GGACAGTGTATATCTGCCAGACCTCTGACCATCTTCAGAATCTCTCTCTGGTCCTAATTGTGAGGGTG  
TGGTGGGGTATTAGCCAGCCACTGCAGGAGGAAGGAGGGCATGGATTGGGATCTTTCTGGTGCAGGAGG  
StRE  
CAAAGGAGTCCAAAAGCCAAGTCAGTGTGTGC**ACAGGGG**AGAGGAGAGAGGCTGAGAGGGGACTAGCACA  
GTCTGGCATGGCTGCTTCAGTCCCTAAATCCTGTTTAGTTTATCATCTAGCTACATCTGCGGCTCCTAGA  
AP-1  
GAACAAGCCAAACACC**AGGAGTCAGAA**ACCCAGCATCAGTAAGCGTGTCTCTGAGAGGTGCATAACCGGA  
C/EBP StRE  
GTCCGTGCCTTAGTCTTTGGGT**ACCC**CAGCTCCCCATGTGAAAAGGGGGACTCCTGTTGTGAGTTTCATC  
TTTCTGGAGACCTTGGAAATGACAGGCTCTAGAGCTGCAGACTTCTCATTATTTCCCCTAAAGTCCTAGAT  
CCTCTGTGAAAGAGCTTGGGATAGGGTACTGGGGAGGTCTGACTTTCTATTACCAGGACTAGAATTCT  
HSE HNF-3b  
GGTTCTGCCATCAATATTGCTGTGTGAC**AAGAAAA**TTCTGGCTTCCCTATTCTCTCATTAGAAATCAAATA  
AP-4  
**GAATATAGATGTCAATGGCAGCTGTACAGATCTTGTGGGCTGCTGTCAGGGTTAGGAGAGCTTTGGCACA**



**Continued (Appendix 5.3.2: Sequence of 4.7 kb of the 5'-region of the mouse HO-1 gene).**

CAGCAAGTGTTCACAAAGGTTGGCTGGGATTGCACCTTTTCGATCAGAGCTTGGTTTCAGCCTTTCTGGC  
StRE  
ACTGTAGTGACATGGAAGCCAGACAGCTGCACCCCATTTCTCAAGGGGAGTCCTGATGCAGCCACTCATTC  
NF - kB  
TGACAGTTATTAAGTCCAGCTACTTGCCAGGCACAGACCCCATCCTGGGGATTTACAAAGCAAAATGAA  
HSE  
CAGTGTCCCTGCCCACCTGCTTTCCAGGTCACCCCGGGGGAAGATGGTCCTTACCAGAAGACCTAGAGAA  
ACACTTGCTTGTGGCAGTGGTCATGGCCACTTTTCCTGCCAATCCATTTCGAAGGCAGAAGATATGCCAC  
CTGCTTTCCAGGGACTGTGAACGTCCTCCTGTTACAGTGAACACATCCTTATCTTAAGCCTCAGTGTCT  
AP-4  
CTGGTTTGTAAAGCCAGCTGATACCTGTACTTCTTTGGTGGAGTTCCCTGAGAGAGTGAAATGAGTTGTCT  
AP-4  
GCATGCCGGGTCTTCCGTTATTATGAGCTGTTATT  
CCATTGTCACCTGCTTCTCGTGGCAGAGAAATGGCAGCTCTCTTTACCGTGTGCCACAGATTCTTGGGTAC  
CREB  
ATGCAGGTGAGACTTGGCCATCCCATTTCCCTCTGTGGGGAATCAACCCTTGATGGGCAGTTGTTGACTT  
CCATTCTCCTTCTCTCAGAGTCGAGGACTTAGAACCACCATTTTCATTTTAGCCTTAAAAACAGAAGAAGA  
AP-1  
GCGAGTCATGGTAGTGCACGCCTTTAATCCCAGCACTTGGGAGGCAGAGGCAGGCAGATCTCTGAGTTTG  
AGTTTTGCCTGGTCTTCTGAGTTTGAGGCCAGCCTGGTCTACAAAGTGAGTTCAAGGACAGCCAGGGCTA  
HNF-3b  
CACAGAGAAACCCTGTTTTTTTTTGGGGGGGGAAGAAAGAAAATATAATGTCCAAAATTAAGTAAATC  
CCTCCTGCTTAGGAACACCAGAGGTAACACCCGAATGCAGGGAGTTGTAGGTCTATGGGAAAGTACTGAG  
StRE NF - kB  
CCTATAGGTTTGTCTTATAAACCCTGAAACATGAGGCTCAAGGCCATTATCAGCTGGGAAATCCAGAA  
HNF-1  
GAACATACCTGGTCAAAGTCCATCCCCAAAGGTTAGTATTTAATATTTAAAGCTTGCTTCTAACTTGGCC  
CAAAATGTTGGAGTTGGTTTTTCTGGAGAATCTCAGGATTAACAAAACAAAGAAACAAAGAATGAAATC  
CREB  
TTTGTGACATCAAAAGTCATTTTGAGGCTGGAGAGATGGCCTGTGGTTGACAGTACTTTCTGCTCTTAC  
StRE  
AGAGGACTTGAATTCAGTTGTGAGCATGGGCAGCTCACTACCACCTTAATTCCCAGTTCTAGGGGATCCA  
HSE  
ATGCCCCTTTCTGGCCTTCTCAGGCACCTATATGCA  
-1260  
C/EBP HNF-3b  
CCCAGCATGAATGCATGCATACATATACATGGGCCTATACACACACATAAAATAAATAATATTC  
HSE  
TTTTTCATTGTTGTCCTTCTCCAGACAGGGTTTCTCTGTGTAACTCTGCTGTCTGGAACCTCACTCTGTAG  
ACCACACTGGTTCCAAACTCACAGAGATCTGCCAGCCTCTGCCTCCTAGGTGCTAGAATTAAA  
HNF-3b  
GGAATGTGCCATCACTACCCAGAAAATAGTAAATACTTTTTTAAAGAAATATTTTAAAGAAGTAAATACTTT  
TAACAACAACAAAAGAGGACATACAAACTATGGAGGCTTTGAAGAACCACCCAGTATGTCTGTGTCTATG  
TCTGTTCTGTACTGAAGAGTCCAGCTCATCAGGAATTCAGAAGGTGAATTCCAAGCCTTACCCTGCTAAG  
CAG  
C/EBP CREB  
CAGTGGGTATCTGTGGTGATAGATGTGTCTCAGAGCTATGTGTCTGACTTCACAGTCTTCTGTACCTGGGA  
MREb MREa  
TCAGGAAGTCATGGTGTGGACCAGGTTCTAGGAGAGCACCAGAGAAGGCAGCCAAACACTCGGGTGCTGCA  
CTCCATGAGACCTGGGCAGAACACTTCAACCTCCAGCTCAGATTCCCCACCTGTAAAACCTGGAGCCATC  
TATGTCCCCACCATGATATATAGGGATAAACCATGGAAGGTTAATCAAGGCAAGTGTGAGCAAAACCAAG  
CAGAAGTGTGAACATAAAGGATTGAAAAAACAGCTATCCTAAGAGTTCCGGGAAGGTAGACTCAGGTAG  
TGTGGGTCAATCCCTGGGTTTGCCACCAGCATCTCACATTTAGGATCGTCTTATAGTTC

[illegible]

-1416

GAATTC CAGGAAG TTTTTTTTTTTTTTTTTT GTTTTTTTTTTTTTTTT GAGGGACAGCGTCTTGTTCTGTGTT  
GCCCAGGTTAGAATACAGTAGCGTGGTCA CAGCTCACTCCAGCCTCTACATCCCAGGCTCAAGTGAACCT  
CCAGCCTCAGCCTCCCAAGTAGCTGGGACCACAGGCATGTGCCACCATGCCCAGCTAATTTATTTTATAT  
TTTGTAGAGACAGGGTCTCCCTATGTTGCCCAGGCCAGTCTCGAACTCAAAGCAATCTTCCCACCTCGAC  
TGGGCTCAAAGCGCTCTTCCCACCTCAACCTCCCAAAGTACTGGGACTACAGGTGTGAGCTACCATGCCA  
GGCCTGAAAGCCATCTTAAAAAAAAAAATCTTAGAATGAGATCACAGTATTGGGAAAGGACTGTATGAATC  
ATCTGGTCCATTTCGTTTGTCTCTGGGTTCACCCAGTGACCCTATTTCCCCGAGTTCTAAGGAGTCCA  
CCTCATGCAGAATTGATTCAATAGGCGATCAGCAAGGGCCAGCTCTGCTCTGGGCCCTGAGCAGGCACTG  
AGTATAAGTCAGACCTGAATGTGCCTGGAAGAGTGTCCACGCATTCCAGCAGGGAAGCAGTTTGTATGA  
CAGGTGTCCCAGTCCAGGCGGATACCAGGTGCTGCCAGAGTGTGGAGGAGGCAGGCGGGGACTTAGTCTC  
CTCCCTGGGTTTGGACACTGGCATCCTGCTTTATGTGTGACACCACTGCACCCCTCTGAGCCTCGGTTTC  
CCCATCTGTAAAAATAGAAGCGATCTACCCTCACAGGTCA GTTGTAGGGATGAACCATGAAAATACTAGAG

*Bgl* 1 HSE

TTCCTGATGTTGCCAC**CAGGCTAT**TGCTCTGAGCAGCGCTGCCTCCCAGCTTT**CTGGAACCTTCTGGGA**  
CGCCTGGGGGTGCATCAAGTCCCAAGGGGACAGGGAGCAGAAGGGGGGGCTCTGGAAGGAGCAAAATCACA

TCATATGACTGCTCCTCTCCACCCACACTGGCCCGGGGCGGGCTGGGCGGGCCCCCTGCGGGTGTTCG

USE TATA

EXON 1

## CHAPTER 6 DISCUSSION

This study originally intended to design new plasmid DNA expression vectors for the purpose of targeted gene delivery. The strategy involved the design of new synthetic promoters/enhancers, upstream of a gene of interest, using regulatory elements associated with the expression of a specific target tissue/organ. The goal of such gene delivery was to correct either an inherited genetic or metabolic disease within a specific target organ (i.e. targeted gene therapy).

Using the computer analyses, the transcription-binding sites associated with the liver (i.e. the target organ) were first identified. Next, from the population of the identified hepatocyte-specific transcription factors (see **CHAPTER 3 Result 1**), HNF4 was chosen for the purpose of the present study.

Based on the mechanisms of stable latent replication and extrachromosomal persistence of the 172 kb human herpesvirus EBV, the episomal EBV-based vector was chosen as the plasmid DNA delivery system. The functional components required for the episomal persistence of EBV-based vectors are the EBV latent origin of replication (*oriP*) present in *cis* and expression of the viral protein EBNA-1. The EBNA-1 protein binds to the family of repeats (*FR*) element, a tandem array of 20 repeats of a 30 bp motif within *oriP*, and this association promotes nuclear retention. Another region of *oriP*, the dyad element, acts as the latent replication origin of EBV. Incorporating the *oriP* elements into plasmid vectors has been shown to allow constructs to remain stably episomal for long periods of time (Yates *et al.*, 1985).

Next a tandem array of 1, 3 or 5 repeats of the 12 bp HNF-4 binding site was inserted upstream of the luciferase gene within the EBV-based vector p706. To allow the expression of the luciferase gene, the SV40 promoter fragment of pGL3 promoter vector (Promega, UK) was inserted downstream of the HNF-4 binding sites. The p706/HNF4/SV40 reporter vectors were then transfected in both liver-specific HepG2 and non-liver HtTA-1 cell lines. The results revealed several unexpected findings:

- Firstly the luciferase expression in cells transfected with p706/SV40 was significantly higher in HepG2 cells when compared with HtTA-1 cells suggesting that SV40 promoter of pGL3 could be used as a liver-preferential promoter, as it stimulates preferentially the transcription of the luciferase gene in a liver-specific cell line. Secondly the computer analysis revealed the presence of a DNA sequence resembling the liver-enriched C/EBP transcription factor-binding site within the SV40 DNA fragment, indicating that this binding site might be a functional site and therefore it can at least in part, contribute to stimulation of the HepG2-preferential expression of the luciferase gene. However this needs to be confirmed for example by performing a DNA bandshift assay that will monitor the formation of a complex between a radio-labelled oligonucleotide containing the C/EBP-resembling sequence of SV40 promoter and the HepG2 protein extracts. Alternatively, a Dnase I foot print assay will reveal whether this element is functional.
- Secondly, the presence of HNF-4 elements decreased significantly the basal level of luciferase activity under the control of SV40 promoter especially in HepG2 cells, suggesting that HNF-4 might act as a repressor. The comparison



of this data with the literature revealed that there are few studies showing that HNF4 could act as a gene repressor (see **INTRODUCTION section 1.1.5.**). For example Chowdhury *et al.* (1996) in an attempt to elucidate the mechanisms governing liver-specific transcription of the liver arginase gene, found unexpectedly that the liver-type arginase promoter activity stimulated by C/EBPs was repressed by another liver-enriched transcription factor HNF-4. The authors speculated that HNF-4 might be involved in fine regulation of the arginase gene in the liver. In the present study, it could be hypothesized that the binding of HNF-4 to the binding sites created within the p706 vector could repress the SV40 promoter activity by suppressing the C/EBP-mediated stimulation of the luciferase activity in HepG2 cells. However further in-depth investigations are necessary to confirm this hypothesis.

In the light of the above data, it was clear that HNF-4 was not an ideal liver-enriched candidate for the liver-directed gene expression. Recent literature within this field reveals more and more that liver-specific genes are regulated in an extremely complicated manner (see **INTRODUCTION section 1.1.5.**). Indeed the previously thought liver-specific *trans*-activators are now shown in several instances to repress the transcription of liver-specific genes that are activated by other liver-enriched transcription factors (Gregori *et al.*, 1993; Gregori *et al.*, 1994, Kritis *et al.*, 1993; Piaggio *et al.*, 1994; Rouet *et al.*, 1995; Chowdhury *et al.*, 1996). In addition there are now numerous examples of studies where liver-enriched transcription factors negatively regulate other genes. An example of negative HNF-4 regulation is the mitochondrial HMG-CoA synthase gene. HNF-4 binds to the mitochondrial HMG-CoA synthase

nuclear receptor response element and represses peroxisome proliferator activated receptor (PPAR)-dependent activation of reporter gene linked to the mitochondrial HMG-CoA synthase gene promoter in HepG2-cotransfected cells (Rodriguez *et al.*, 1998). Another example of negative regulation by HNF-4 is the acyl-CoA oxidase gene. Both PPAR $\alpha$  and HNF-4 efficiently bind to the acyl-CoA oxidase gene enhancer element, but PPAR $\alpha$  exhibits much stronger transactivation than HNF-4. As a result, HNF-4 suppressed the gene-activating function of PPAR $\alpha$ , when they were expressed together, due to competition for a common binding site (Nishiyama *et al.*, 1998).

Furthermore, liver gene regulation studies have illustrated basic principles observed for other cell differentiation systems i.e. *cis*-regulatory systems are modular, typically including a cluster of binding sites for both cell-restricted and ubiquitous transcription factors. None of the cell-specific factors are restricted to hepatic cells, indicating a more complex mode of action, in which a unique combination of regulators, often working synergistically, defines a particular phenotype. The simultaneous presence of multiple transcription factors may also be required in the response to extracellular signals. This combinatorial mode of regulation is not specific to the liver, but rather it is a common theme in many mammalian cell differentiation systems (Cereghini, 1996). Another common feature is that a single *cis*-acting element can interact with a family of transcription factors displaying similar DNA binding specificity, often coexisting in the same cell. Conversely, single transcription factors appear to serve multiple developmental functions in the organism (see **INTRODUCTION section 1.1.5**). Moreover, the organization of the chromatin structure and its reorganisation during development, certainly play a critical role in gene activation. Studies with transgenic

mice have shown that while relatively small promoter regions define liver-specificity and hormonal regulation in cultured cells, often distal enhancers are required to reproduce the in vivo endogenous expression (e.g. Cuif *et al.*, 1992). The homo- and heterodimerization between members of a particular transcription factor family adds another level of complexity not yet understood (see **INTRODUCTION section 1.1.5.**). Additional determinants of specificity that remain to be further investigated include protein-protein interactions, cell-specific coactivators or cofactors, protein modifications, competitive interaction or synergy of factors bound nearby or in close.

In the second part of this thesis, efforts were concentrated on identification of a gene which, was expressed at unusually high level in the target tissue (liver) and then to use the promoter for this gene to drive the expression of the luciferase reporter gene to mimic a therapeutic gene in the target tissue. Since HO-1 gene is known to be highly expressed in the liver, it was logical to use the promoter of this gene for the purpose of this study. In addition the p706 reporter constructs that were obtained from the Prof. Tyrrell's laboratory contained originally the 4.7 kb of the human HO-1 5'-flanking region (i.e. plasmid p706/HO4.7). In addition this laboratory possessed an array of p706/HO derived plasmids that was constructed following a series of sequence deletions within the 4.7 kb of the 5'-flanking region linked to the luciferase gene.

The first step was to explore the basal regulation of the gene expression to gain insight into the mechanism of tissue-preferential HO-1 gene regulation. To identify the *cis*-acting elements that regulate basal human HO-1 gene expression, reporter plasmids containing deletions within the specific HS regions of 4.7 kb of the human HO-1 5'-

flanking region linked to the luciferase gene in p706 vector were transiently transfected into human carcinoma cell lines HepG2 and Hela cells. Since the 5'-flanking region of human HO-1 is not as well studied as in mouse, a data base program called TFSEARCH was used to locate the transcription factor elements in the 4.7 kb of the 5'-flanking region. The transient transfection assays confirmed that the expression of the luciferase reporter in p706/HO4.7 construct is preferentially higher in HepG2 cells when compared to the non-liver cell line HtTA-1. Furthermore it appeared that the HS-3 region (position -4080 to -3650) that contains the region homologous to SX2 mouse enhancer is critical for such tissue preferential expression of luciferase in HepG2 cells. This region contains the previously identified human Cd<sup>+2</sup>RE. (Takeda *et al.*, 1994). Furthermore the computer analysis reveals that this region contains sequence resemblance to several critical *cis*-acting elements such as the liver enriched C/EBP $\beta$ , as well as important elements such as NF-E2 and AP-1a and AP-1 b (see **Appendix 5.3.2. CHAPTER 5 Result 3**). Also the comparison of p714 mutant construct with p717 and p718 strongly suggest that the region -380 to +1 where the HS-1 and HS-2 regions are located contains liver-preferential negative regulatory elements. The precise nature of these negative regulatory elements need to be investigated by methods such as bandshift and Dnase I foot print or chromatin immunoprecipitation (chip) assay. However within the context of the present study, this negative regulatory region coincided with the HS-2 region containing the highly polymorphic (GT)<sub>n</sub> repeats, that is known to negatively regulate the gene transcription (Yamada *et al.*, 2000). So one possibility is that these dinucleotide repeats influence the expression of luciferase in the p714-transfected cells as these dinucleotides repeats are thought to act as a repressor of the human HO-1

transcriptional activation (Kimpara *et al.*, 1997). Furthermore, since the number of these repeats appears to influence the level of modulation of gene expression, the GT repeats within the 4.7 kb fragment was carefully monitored following sequencing and was found to be around 29 GT repeats. This number GT repeats could be classified as class 'M' (25-29 repeats, medium) according to the study by Yamada *et al.* (2000). The recent study by Takahashi *et al.* (1999) that assessed the 4 kb of the 5'-flanking region of the human HO-1 for basal activity in both HepG2 and Hela cells, does not report such a negative regulatory region within the first 380 bp upstream promoter region of HO-1 presumably because of the low number of such GT repeats in their constructs. Indeed, the sequencing performed by these authors (i.e. approximately 2 kb of the 5'-flanking region) reveals that the number of GT repeats within their constructs is only around 23 that according to the classification by Yamada *et al.* (2000) falls into the category 'S' (small, < 25 repeats). Takahashi *et al.*, (1999) also reported a positive regulatory region at position -1976 to -1655 bp that functioned only in HepG2 cells but not in Hela cells. Unfortunately because of the original design of the p706/HO vectors in Prof. Tyrrell's laboratory (constructs considering the role of HS regions in HO-1 expression), it was not possible to compare this data with the present study as this region was consistently deleted in the p706/HO mutant constructs. The only construct that contained this region was p736, but this construct also contained more than 3 kb undeleted sequence when compared to other mutant constructs. So it is clear that further analyses are necessary to draw any conclusion concerning this positively regulated region.

Overall, it appears that the 4.7 kb of the 5'-flanking region of human HO-1 can be used as a liver-specific promoter/enhancer sequence to drive expression of other genes in the liver. However further detailed functional analyses are necessary to understand the liver-HO-1 gene regulation and also to identify the most liver-specific *cis*-acting elements within the 5'-flanking region of human HO-1 gene.

### **6.1. Future Work**

As mentioned previously, liver-specific genes are regulated in an extremely complicated manner. So it is hard to design functional synthetic specific promoters for tissue-targeted gene delivery. Although the approach used in this study was carefully planned, the outcome of the data clearly indicated that choosing a single liver-enriched transcription factor binding site even in tandem repeat is overenthusiastic. The future project should therefore consist of using the data obtained from the TFSEARCH analyses of the present study in order to select a group of transcription factor binding elements for the design of synthetic liver-enriched promoters. A recent study by Li *et al.*, (1999) has described a strategy for the construction and characterization of synthetic muscle promoters, whose transcriptional potencies in terminally differentiated muscle greatly exceeded those of both the natural myogenic skeletal  $\alpha$ -actin gene promoter and viral promoters. This has been achieved by random assembly of specific muscle-specific transcription binding sites into synthetic promoter recombinant libraries, and screening of hundreds of individual clones for transcriptional activity *in vitro* and *in vivo*. A similar approach should be applied to continue the present study by for

example random assembling different combinations of liver-enriched HNF-1, HNF-3, HNF4, HNF6, C/EBP and DBP transcription factor binding sites.

The HO—1 sequence deletion mutants in the present study were designed around the role of HS sites. However in order to elucidate the regulation of the human heme oxygenase-1 (HO-1) gene expression as an attempt to understand the mechanism of hyperoxic and tissue-preferential HO-1 gene regulation, a new series of progressive deletion mutants of the 5'-flanking region linked to a reporter gene has to be constructed. Also it is necessary to use more liver- and non-liver specific cell lines as models to obtain the true comparison between the *cis*-acting regulatory elements that are liver-specific and those elements that are not. The (GT)<sub>n</sub> dinucleotide repeat in the 5'-flanking region of human HO-1 gene shows length polymorphism and appears to modulate the level of liver specific gene expression. So further investigations could include analysis of the number of these repeats through either a series of small scale deletions within this region or through insertion of synthetic oligonucleotides containing additional repeats.

Furthermore, most of the researchers have used approximately 4 kb of the 5'-flanking region of the human HO-1 gene to assess the promoter activity. Bioinformatic research analysis database program TFSEARCH could be used to allocate the transcription factor elements within the 10-13 kb of the 5' flanking region. Bandshift and DNase I footprint assays could then be carried out to further identify these sites. It will also be important to clone the 10-13 kb upstream region in p706-based EBV-vectors to identify the critical liver-specific region within the 5'-flanking of human HO-1 gene.

Furthermore the previous identification of HS-sites was performed with the method developed by Wu (1980). This method lacks precision, so the positions of each HS site are an approximation. Recently McArthur *et al.*, (2001) has designed a precise methodology that allows the quantification of the sensitivity of chromatin to digestion by Dnase I using real-time PCR. This approach has three clear advantages over the more conventional use of Southern hybridization assay: (a) the accuracy of quantification is improved; (b) the resolution of the assay is enhanced, by designing primers to amplify small amplicons it is possible to analyse sequences both coincident and proximal to sites of Dnase I hypersensitivity, (c) less material is needed (i.e. 5ng of treated genomic DNA).



## 7. REFERENCES

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